Identifying infection reservoirs of digital dermatitis in dairy cattle

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

Jennifer Bell

September 2017

Table of Contents

Abstract

Identifying infection reservoirs of digital dermatitis in dairy cattle – *Jennifer Bell*

Digital dermatitis (DD) is an infectious ulcerative dermatitis typically affecting the skin of the hind feet of dairy cattle worldwide with substantial welfare and economic implications making it an important issue for the dairy industry. A polytreponemal aetiology has been described with three distinct cultivable treponeme phylogroups (*Treponema medium, Treponema phagedenis* and *Treponema pedis*) consistently detected within DD lesions in the UK and USA. Current control strategies are failing to eliminate DD on farm and there is little known about DD transmission. Identifying the infection reservoirs of DD treponemes would inform new targeted prevention strategies for DD.

Dairy cattle gingiva, recto-anal junction (RAJ) and DD-unaffected foot tissue (previously identified as potential infection reservoirs) along with samples from the dairy farm environment were surveyed for the presence of DD treponemes by molecular and cultivation techniques to determine their role as infection reservoirs. DD treponemes were detected in 14/122 gingiva sampled, 2/121 RAJ sampled and 41/217 DD-unaffected feet. No temporal association with presence of DD treponemes in these tissue types was identified. Detection of DD treponemes in dairy cattle faeces (*n*=62*),* mucin casts (*n*=31), water (*n*= 19) and feed samples (*n*=36) failed by PCR, despite use of optimised detection techniques for DD treponemes in faecal material. However, for the first time a treponeme belonging to the *T. phagedenis* DD treponeme phylogroup was isolated from a dairy cattle faecal culture. In addition, a second faecal culture was also positive by PCR for the *T. phagedenis* DD treponeme phylogroup.

DD treponemes were detected on dairy cattle fomites with 9/16 foot trimmer gloves positive for DD treponemes which were only detected following DD-affected foot handling. DD treponemes were also detected on a small number of foot trimming tools other than the foot trimming knife blades. Additionally, for the first time, DD treponemes were detected in 22/169 dairy cattle footprints, with the largest proportions detected in footprints on concrete and rubber floors.

Further investigation into the carriage of DD treponemes in tissues using histopathology and immunohistochemistry (IHC) on tissues demonstrated that gingiva and RAJ tissue had no signs of disease; however, almost all healthy foot tissue (PCR positive for DD treponemes) appeared to have changes in the tissue associated with infection. Multi-locus sequence typing (MLST) revealed that the same sequence types of the DD treponeme phylogroups found in DD lesions of various species could also be found in gingiva, RAJ and DD-unaffected foot tissue of dairy cattle.

Survival of DD treponemes was assessed in a range of different conditions, with DD treponemes remaining viable when cultured between the pH values of 5.5 and 9 and in temperatures of 4-37 °C under anaerobic conditions. DD treponemes remained viable in faecal microcosms incubated aerobically for a median of 1 day (range of 0-6 days). In five different bedding microcosms under aerobic conditions, DD treponemes were viable for the full 7 days of the study in sand bedding, for 6 days in sawdust and for 5 days in recycled manure solids (RMS). However, DD treponemes were not viable at any time point when inoculated into bedding microcosms of straw or sand containing 5% (w/w) lime.

In conclusion, these studies have demonstrated that DD treponemes have a diverse range of potential infection reservoir sites including the gastrointestinal (GI) tract and fomites, and along with survival information, this knowledge can be applied to the development of preventative measures to mitigate DD transmission.

Acknowledgements

Thank you to my funders, AHDB Dairy, for their funding, support for this project and for the advice to investigate ‘green bedding’.

A large thank you to my supervisors, Dr Nick Evans and Prof. Stuart Carter, for their never ending knowledge on all things spirochaete and their patience and support throughout the completion of this project. Additional thanks to my secondary supervisor Dr Richard Murray for enabling my frequent trips to collect tissue samples.

A big thank you to everyone I was lucky enough to work with as part of the treponeme infection team: Dr Simon Clegg, Dr Leigh Sullivan, Kerry Newbrook, Dr Stu Ainsworth, Gareth Staton, Intan Aina Kamaruzaman and Dr Jennie Mullin. Their enthusiasm for all things DD was infectious and they made the experience a lot of fun.

A special thank you to Simon Clegg, for putting up with my constant questions and food related grumps, teaching me sequencing, MLST and all things in between, for always offering to help, but most of all for coming with me to collect samples and going above and beyond to help me get the particular samples I wanted.

I would like to say a huge thank you to Dr Roger Blowey, whose brilliant ideas made some of the most interesting finds in this project.

Thank you to the technical team; Jenna Dawson, Cathy Glover and Catherine Hartley for all their help and support. Particularly Jenna Dawson for her work on Chapter 3.

Thank you to Hayley Crosby-Durrani for all her work and support, especially with the immunohistochemistry in Chapter 5.

To Stuart Ainsworth, thank you for providing the laughs, the freddos and keeping me going when science was not my friend.

To Kerry Newbrook, Lisa Luu and Tess Walsh thank you for your friendship, support, shared love of many things and understanding that food is everything.

To my family, both human and furry, for loving and supporting me, even when I couldn’t come home very often due to lab commitments or play ball because I was writing. Especially thank you to Bailey for keeping me sane.

Thank you to the faves who put up with my absence from many events so I could carry out this project.

Finally, thanks to the person who made the LoTR soundtrack on Spotify which has seen me through writing this thesis.

List of knowledge exchange contributions relating to this thesis

Oral presentations

Bell J, Carter SD, Blowey RW, Clegg SR, Murray RD & Evans N. Identifying infection reservoirs of digital dermatitis in dairy cattle. 7th International Conference on Colonic Spirochaetal Infections in Animals and Humans. Hannover, Germany, 5th-7th October 2016.

Bell J, Carter SD, Murray RD & Evans N. Identifying infection reservoirs of digital dermatitis in dairy cattle. Agriculture and Horticulture Development Board Livestock PhD Seminar. Warwickshire, UK 14th-15th November 2016.

Bell J, Carter SD, Murray RD & Evans N. Identifying infection reservoirs of digital dermatitis in dairy cattle. Agriculture and Horticulture Development Board Livestock PhD Seminar. Warwickshire, UK 9th-10th December 2014.

Bell J, Carter SD, Murray RD & Evans N. Identifying infection reservoirs of digital dermatitis in dairy cattle. Agriculture and Horticulture Development Board Livestock PhD Seminar. Warwickshire, UK 10th-11th December 2013.

Poster presentations

Bell J, Carter SD, Murray RD, Clegg SR & Evans N. Identifying infection reservoirs of digital dermatitis in dairy cattle. The Microbiology Society Annual Conference 2016. Liverpool, UK, 21-24 March 2016.

Bell J, Carter SD, Murray RD & Evans N. Identifying infection reservoirs of digital dermatitis in dairy cattle. Agriculture and Horticulture Development Board Livestock PhD Seminar. Warwickshire, UK 7th-8th December 2015.

Bell J, Clegg SR, Murray RD, Blowey RW, Carter SD & Evans N. Identifying infection reservoirs of digital dermatitis in dairy cattle. 69th Association for Veterinary Teaching and Research Work Annual Conference. London, UK, 2-3rd September 2015.

Bell J, Carter SD, Murray RD, Clegg SR, Sullivan LE & Evans N. Identifying infection reservoirs of digital dermatitis in dairy cattle. Total Dairy Seminar. Gloucestershire, UK, 10th-11th June 2015.

Bell J, Carter SD, Murray RD, Blowey RW, Clegg SR, Sullivan LE, M^{c}Kown R & Evans NJ. Identifying infection reservoirs of digital dermatitis in dairy cattle. Cattle Lameness Conference. Worcester, UK, 7th May 2014.

Abbreviations

Abbreviation	Expanded form
1D	1-Dimensional
adK	Adenosine kinase gene
BIS-TRIS	2,2-Bis(hydroxymethyl)-2,2',2''-nitrilotriethanol - 2-Amino-2-hydroxymethylproprane-1,3-diol
BLAST	Basic local alignment search tool
BN	Norgen Biotek Stool DNA Isolation kit
bp	Base pair
CB	Crude boiling method
CODD	Contagious ovine digital dermatitis
DD	Digital dermatitis
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphates
EGTA	Ethyleneglycol tetraacetic acid buffer
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
FAA	Fastidious anaerobic agar
FCS	Foetal calf serum
FISH	Fluorescence in situ hybridisation
g	Grams
gdh	Glutamate dehydrogenase gene
GI	Gastrointestinal
Glouc	Gloucestershire

glpK	Glycerol kinase gene
groEL	Heat shock protein gene
IHC	Immunohistochemistry
kDa	Kilodalton
kg	Kilogram
L	Litre
LAMP	Loop-mediated isothermal amplification
mA	Milliamp
MALDI-TOF MS	Matrix assisted laser desorption ionisation time-of-flight mass spectrometry
MBC	Minimum bactericidal concentration
MEGA	Molecular evolutionary genetics analysis
mg	Milligram
MIC	Minimum inhibitory concentration
ml	Millilitre
MLST	Multi-locus sequence typing
mM	Millimolar
MP	MO BIO PowerSoil® DNA Isolation Kit
N/A	Not applicable
NT	Not tested
OD	Optical density
OTEB	Oral treponeme enrichment broth
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tween
PCR	Polymerase chain reaction
PF	MO BIO PowerFecal® DNA Isolation Kit
PW	Promega Wizard® Genomic DNA Purification Kit

pyrG	Orotidine 5'phosphate dehydrogenase gene
qPCR	Quantitative polymerase chain reaction
QS	Qiagen QIAamp® Fast DNA Stool Mini Kit
QT	Qiagen DNeasy® Blood and Tissue Kit
RAJ	Recto-anal junction
rcf	Relative centrifugal force
recA	Recombination protein A gene
rplB	Large polymerase sub unit gene
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RS	Rabbit serum
SDS	Sodium dodecylsulphate
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
ST	Sequence type
TAPS	Tetraacetylphytosphingosine
TEMED	Tetramethylethylenediamine
Tris	2-Amino-2-hydroxymethylproprane-1,3-diol
V	Volts
v/v	Volume/ volume
UK	United Kingdom of Great Britain and Northern Ireland
Worcs	Worcestershire
w/v	Weight/ volume
w/w	Weight/ weight
µl	Microlitre
µM	Micromolar

Chapter 1: Introduction

1.1 Bovine digital dermatitis – overview

Digital dermatitis (DD) also known as hairy heel wart, strawberry foot, Mortellaro's disease or papillomatous digital dermatitis is an important disease that has emerged over the past 40 years in dairy cattle and continues to blight the dairy industry. Initially reported in dairy cattle in 1974 in Italy, DD is now a global disease of the dairy industry and was first reported in the UK in 1988 in which it is now endemic (Blowey and Sharp, 1988). DD presents as an ulcerative dermatitis typically affecting the digital skin on the plantar aspect of the hind foot between the bulbs of the heel, above the interdigital cleft (Cheli and Mortellaro, 1974; Blowey and Sharp, 1988; Read and Walker, 1998). These lesions are detrimental to cattle health and welfare and have serious economic implications for the dairy industry. Forms of the disease have now been discovered in beef cattle (Sullivan et al., 2013), sheep (contagious ovine digital dermatitis, CODD) (Harwood et al., 1997; Davies et al., 1999; Dhawi et al., 2005), elk and goats (Clegg *et al.*, 2015; Sullivan *et al.*, 2014a).

DD is an infectious disease and a polymicrobial aetiology has been described for DD with spirochaetes belonging to the *Treponema* genus strongly implicated as the primary causative agents in lesion development and progression (Choi et al., 1997; Stamm et al., 2002; Evans et al., 2008, 2009c; Klitgaard et al., 2008). Despite over 40 years of research the transmission routes of DD remain elusive. With challenges such as poor efficacy, antimicrobial resistance and toxicity affecting treatment and control strategies, it would appear that preventative rather than reactive action may be key to DD control on farm. It is imperative that the infection reservoirs and transmission of DD-associated treponemes are understood so that preventative measures are targeted and thus are more likely to make an impact on DD.

1.2 Clinical manifestations of DD

1.2.1 Anatomical location of DD lesions

The lesions of DD are usually found on the plantar aspect of the rear feet, between the bulbs of the heel directly above the coronet (Figure 1.1) (Cheli and Mortellaro, 1974;

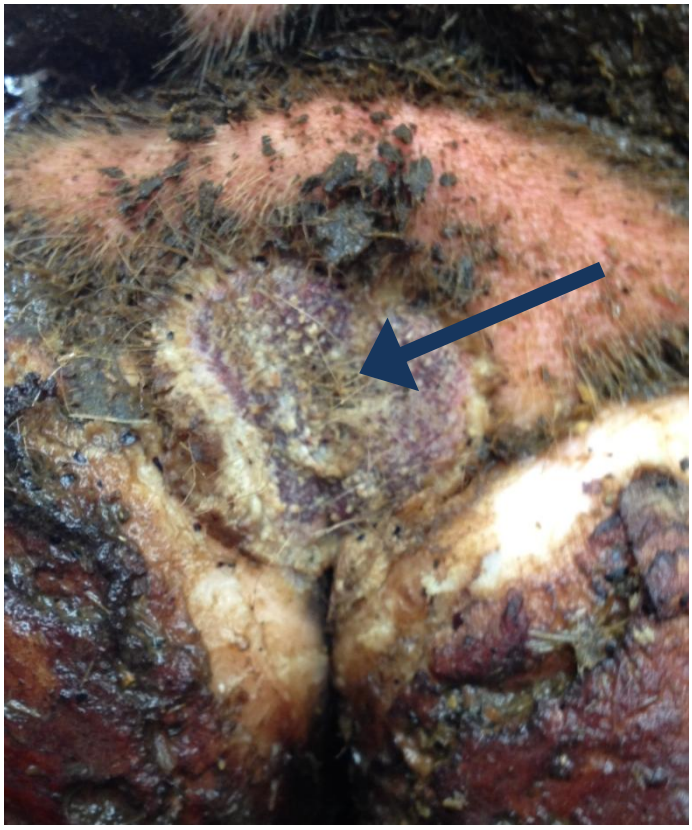
Blowey and Sharp, 1988). However, occasionally DD lesions have been observed on the skin of the interdigital cleft, the junction between skin and horn on the heel, the dew claw and dorsally on the coronary band (a vertical wall crack may be associated with this localisation) (Read and Walker, 1998; Dopfer, 2009; Shearer et al., 2016). A small proportion (approximately 10-20%) of DD lesions have also been observed on the front feet (Blowey and Sharp, 1988; Read and Walker, 1998; Murray et al., 2002). There may only be one DD lesion present or multiple DD lesions present on a single foot (Read and Walker, 1998). Additionally, DD may affect only one hind foot or both hind feet at any one time with studies finding approximately 30-50% of feet concurrently affected (Laven, 1999; Holzhauer et al., 2006).

1.2.2 Clinical appearance and histopathology of DD lesions

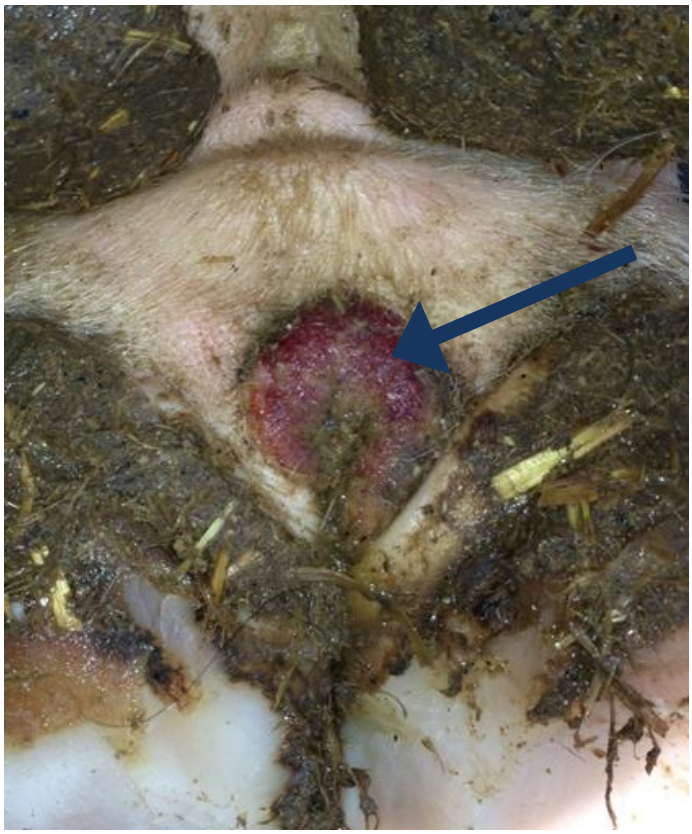
DD lesions present as an irregular circular or oval area (between 1 and 6 cm) of circumscribed or diffuse ulcerative dermatitis in which the lesion may appear red (Strawberry-like) or a white-yellow-grey blend (Blowey and Sharp, 1988; Read and Walker, 1998). The lesion may appear moist with exudate, bleeding may be observed and the smell is repugnant (Cornelisse et al., 1981; Blowey and Sharp, 1988; Read and Walker, 1998). As lesions progress and become chronic they can develop proliferative papillary projections (Read and Walker, 1998). Lesions are often painful upon touch and can sometimes result in severe lameness in which cows walk on their toes; often resulting in further damage to the foot (Blowey and Sharp, 1988; Bassett et al., 1990; Read and Walker, 1998). However, lameness does not always accompany the development of DD lesions.

Briefly, the histopathology of DD lesions show the epidermis may be thickened with the stratum corneum experiencing parakeratotic and/ or orthokeratotic hyperkeratosis (Dopfer et al., 1997; Manske et al., 2002a; Cruz et al., 2005; Rasmussen et al., 2012; Nielsen et al., 2016). Irregular erosion of the stratum corneum is frequently observed or the epidermis may be completely lost as a result of ulceration (Dopfer et al., 1997; Krull et al., 2014). Micro-abscesses are detected at the tips of the dermal papillae following pronounced retes peg formation (Dopfer et al., 1997; Manske et al., 2002a; Cruz et al., 2005; Rasmussen et al., 2012; Nielsen et al., 2016). Ballooning degeneration of keratinocytes is visible along with reactive inflammation in which there are large infiltrates of inflammatory cells (Dopfer et al., 1997; Manske et al., 2002a; Rasmussen et al., 2012; Nielsen et al., 2016). Progression of the lesion results in increasingly worsening changes of the histopathology (Dopfer et al., 1997).

a)



b)



c)

Figure 1.1: Examples of DD lesions

Figures a), b) and c) show DD lesions present on the skin of the plantar aspect of the hind foot, between the bulbs of the heel and above the coronet. Figure a) depicts an M2 (active ulcerative) stage DD lesion (arrow) which is greater than 2 cm in diameter with a red-grey mottled appearance. Figure b) depicts an M2 (active ulcerative) stage lesion (arrow) which is greater than 2 cm in diameter appearing very red and moist. Figure c) appears as a mixed stage lesion with an M2 (active ulcerative) lesion (blue arrow) similar in appearance to figure a) however also shows signs of the chronic M4 stage (green arrow) showing thickening of the epithelium. Figure a) and c) author's own photographs. Figure b) gifted by Roger Blowey, Wood Veterinary Group, Gloucestershire.

1.2.3 DD lesion classification

Over the course of a DD infection the lesions progress through clear stages in which the appearance of the DD lesions change. There are different methods of defining (or scoring) these disease stages (Dopfer et al., 1997; Laven, 1999; Manske et al., 2002a; Vink, 2006; Krull et al., 2014), however, the most prominently used system is the M stage classification system (Dopfer et al., 1997; Berry et al., 2012; Holzhauer et al., 2012). The M stage classification system defines DD lesions into six different stages M0-M4.1 based upon macroscopic appearance. Briefly, M0 refers to a normal DD-unaffected foot; M1 is described as an early lesion and is characterised by a small less than 2 cm circumscribed

lesion which may appear red/ grey, moist and ragged; M2 is the active ulcerative stage of the lesion (greater than 2 cm), often strawberry like in appearance and can be painful to touch (Figure 1.1); M3 is where the lesion is healing and a brown scab, firm but rubbery in texture is present; M4 denotes a chronic lesion (Figure 1.1c) showing dyskeratosis and/ or proliferative growth which may form papillary projections; M4.1 is still a chronic lesion, however, there are small foci of M1 lesions developing (Dopfer et al., 1997; Berry et al., 2012; Döpfer et al., 2012b; Holzhauer et al., 2012).

1.3 Epidemiology of DD

1.3.1 Geographic spread of DD

Bovine DD was first reported in dairy cattle in Italy in 1974 and has since become a worldwide problem for dairy cattle (Cheli and Mortellaro, 1974). It was later reported in multiple states of the USA (Rebhun et al., 1980; Rodríguez-Lainz et al., 1996; Read and Walker, 1998; Brown et al., 2000), the UK (Blowey and Sharp, 1988), Japan (Kimura et al., 1993), South Africa (van Amstel et al., 1995), Mexico (Argáez-Rodríguez et al., 1997), Chile (Rodriguez-Lainz et al., 1998), Switzerland (Luginbuhl and Kollbrunner, 2000), Egypt (el-Ghoul and Shaheed, 2001), Brazil (Cruz et al., 2001), Sweden (Manske et al., 2002b), New Zealand (Vermunt and Hill, 2004) Germany (Koenig et al., 2005), Netherlands (Holzhauer et al., 2006), Denmark (Capion et al., 2009) and France (Relun et al., 2013b). In many of these countries, including the UK, DD is now endemic.

1.3.2 Prevalence

In the UK, it is estimated that DD can be found in over 70% of dairy herds (Laven, 2001). Within herds the rate of DD can vary greatly, which is likely due to different farm practices. Studies have found smaller herds have a lower prevalence of DD (Holzhauer et al., 2006; Barker et al., 2009), for example, one study found that a small herd of less than 79 cows had a 5% rate of DD per 100 cow-months (months of lactation) whereas the larger herd size of over 160 cows had a DD rate of 52% per 100 cow-months (Barker et al., 2009). In the Netherlands a cross-sectional survey (383 herds) found that 91% of herds surveyed had DD and within herd prevalence was typically between 5-10% although the data ranged from 0 to 83% (Holzhauer et al., 2006). Similarly in Chile, 91% of the 43 dairy farms surveyed had DD and within herd prevalence was a median of 6% (Rodriguez-Lainz et al., 1998). Read and Walker, 1998 demonstrated an increase in herds affected by DD between 1991 and 1994 from 31% to 89%.

Recent prevalence figures for DD are difficult to source and to the best of the author's knowledge there are no recent studies in the UK which have endeavoured to investigate this figure. This has lead to the use of largely outdated prevalence figures or figures based on conjectures which may not be a true representation of the prevalence of DD today. An example of which is the commonly cited UK DD prevalence estimate of 70% of herds (Laven, 2001) described above. Whilst the figures described above can give a limited understanding of the scale of the DD problem for the dairy industry; knowing the current prevalence of DD is key to understanding the dynamics of the disease as well as determining the efficacy of DD control strategies currently used.

1.3.3 Seasonality

The prevalence of DD appears to display some seasonality with a tendency for prevalence to be higher in the winter months when cattle would typically be housed although more recent studies are needed to reflect current farm management practices. One study noted an increase in lameness as a result of DD following the rainy season in January-March (Read and Walker, 1998). Another study showed there was statistically significant association (P= < 0.001) of foot skin lesions (including DD) in the winter months (November-April) with 72% of DD lesions observed occurring in the winter (Murray et al., 1996). In addition, a study found that prevalence of DD was highest in the winter months of November-January and lowest between June and July (Vink, 2006). Furthermore it was observed that DD prevalence decreased towards the end of the housing season and began to increase before the grazing season had finished (Vink, 2006). Furthermore in a study investigating seasonality of lameness treatment, the level of seasonality of DD had decreased from 72% of DD reported in winter in previous studies to 60% reported in winter in this study (Laven et al., 2006). However, this is still a higher proportion in winter than summer. It is postulated that the seasonality of DD is likely due to hygiene and close contact of housed cattle during the winter months (Blowey and Sharp, 1988).

1.4 The implications of DD

1.4.1 Welfare of affected cattle

The welfare of an animal with DD is severely compromised. DD lesions can be extremely painful which can result in lameness. Lameness can be described as a change in the gait of an animal due to an abnormality in the animal which may result from disease, environment or management factors (AHDB Dairy, 2016a). DD is one of the leading causes of lameness

and is believed to account for 8-20% of all lameness cases in the UK (Murray et al., 1996; Blowey, 2005). Lameness is often considered a result of pain as the animal tries to take weight off the affected limb. Indeed, behaviours such as resting a hind foot and lifting a hind foot with reluctance to place it back on the floor were shown to be significantly increased in cows with DD than cows with no lesions (Stokes, 2011).

Pain associated with lameness and/or foot disorders can further impact dairy cattle welfare by influencing the cows behaviour resulting in behaviours such as increased lying times (Ito et al., 2010; Navarro et al., 2013), reduced exploratory behaviour (Stokes, 2011) and less time eating (González et al., 2008; Miguel-Pacheco et al., 2014). Changes in behaviour can create opportunities for other diseases or conditions to take hold; for example increased lying time is a risk factor for mastitis (DeVries et al., 2010; Watters et al., 2013), an important inflammatory infection affecting the udders. Additionally, cows may also experience reduced milk yield and fertility as a consequence (Argáez-Rodríguez et al., 1997; Hernandez et al., 2001, 2002).

Whilst lameness can be a key indicator of a problem with the cow, leading to inspection and treatment DD lesions have been shown to be painful without the occurrence of lameness (Laven and Proven, 2000; Manske et al., 2002b; Dyer et al., 2007). Indeed, a study reported pain upon light pressure applied to DD lesions for 90% of cattle tested whereas only 23% of these cows were classed as lame (Laven and Proven, 2000). The cow as a prey animal is known for being able to mask pain (Dyer et al., 2007), thus regular inspection of feet regardless of lack of lameness is important for improving cattle welfare.

Furthermore, the negative welfare impact of a disease is tied to the level of pain and the duration of the disease and for this reason DD in dairy cattle has been shown through a modelling approach to have the highest impact on welfare when compared to seven other foot disorders (Bruijnis et al., 2012). This is especially important due to the chronic, reoccurring nature of DD as a disease.

1.4.2 Economic impact

The economic impact of DD on the dairy industry is considerable as a result of treatment costs, labour costs, reduction in milk yield and reproductive performance as well as premature culling (Argáez-Rodríguez et al., 1997; Hernandez et al., 2001, 2002; Warnick et al., 2001; Bruijnis et al., 2010; Relun et al., 2013b).

In the UK, the cost of DD per case is estimated by AHDB dairy to be UK£98.79 using a customisable cost calculator considering factors associated with reduced milk yield, reduced fertility, treatment costs (including repeat treatments and labour) and culling costs (Willshire and Bell, 2009; GB Cattle Health and Welfare Group, 2014). However, it is unknown what data was used to derive this cost estimate. Another model calculated the average cost of DD per case to be US$132.96 which can be broken down into milk loss costing US$35.41 (27%) per case, decreased fertility costing $41.37 (31%) per case and treatment costing US$56.18 (42%) per case (Cha et al., 2010).

The costs described using models/ calculators are likely to be an underestimation of the costs of DD as a limited number of factors are taken into account and they don't necessarily include secondary diseases that may result from DD, costs of changes to herd management whilst cases are dealt with, DD control measures (e.g. footbathing) are not often accounted for and nor are body condition losses and reduced milk quality (Willshire and Bell, 2009). Additionally, the costs described are taken to be representative of what may be expected of a 'typical' herd and therefore costs are likely to vary between farms with different incidence rates of DD as well as treatment practices. The cost of DD will also have a significant economic impact when the chronic, reoccurring nature of the disease is taken into account. Furthermore once established on a farm DD becomes endemic and will be a constant financial burden.

1.5 The aetiology of DD

1.5.1 A polymicrobial disease

Understanding of the aetiology of DD is still evolving. The quick spread of DD amongst and between herds along with resolution following antibiotic treatment suggested that DD was an infectious bacterial disease (Blowey and Sharp, 1988; Read et al., 1992; Read and Walker, 1998). Investigations into the microbial aetiology of DD quickly established that DD was a polymicrobial disease with a plethora of bacteria present but viral and fungal pathogens were not associated (Rebhun et al., 1980; Krull et al., 2014). Bacterial genera that have been identified in lesions are described in Table 1.1, briefly they include but are not limited to: *Camplyobacter* (Dopfer et al., 1997), *Fusobacterium* (Koniarová et al., 1993), *Bacteroides*, (Koniarová et al., 1993), *Guggenheimella* (Strub et al., 2007; Schlafer et al., 2008), *Porphyromonas* (Berry et al., 2010), *Prevotella* (Berry et al., 2010), *Treponema* (Choi

et al., 1997; Stamm et al., 2002; Evans et al., 2008; Klitgaard et al., 2008), *Mycoplasma* (Berry et al., 2010) and *Dichelobacter* (Blowey et al., 1994b; Rasmussen et al., 2012).

Table 1.1: Microorganisms detected in bovine DD lesions

Bacterial genera[a]	Method(s) of detection[b]	References
Acholeplasma (ales)	Metagenomic sequencing	(Zinicola et al., 2015b)
Alkaliphilus (crotonatoxidans)	Metagenomic sequencing	(Zinicola et al., 2015b)
Bacteroides	Culture isolation	(Koniarová et al., 1993)
Blautia (hansenii)	Metagenomic sequencing	(Zinicola et al., 2015b)
Campylobacter	Immunofluorescence test, IHC, metagenomic sequencing	(Dopfer et al., 1997; Cruz et al., 2005; Krull et al., 2014; Zinicola et al., 2015b)
Candidatus Amoebophilus (asiaticus)	Metagenomic sequencing	(Zinicola et al., 2015b)
Candidatus Blochmannia (rufipes)	Metagenomic sequencing	(Zinicola et al., 2015b)
Candidatus Phytoplasma (prunorum)	Metagenomic sequencing	(Zinicola et al., 2015b)
Clostridium	Culture isolation, metagenomic sequencing	(Koniarová et al., 1993; Zinicola et al., 2015b)
Dichelobacter (nodosus)	Culture isolation, PCR, FISH, metagenomic sequencing	(Rasmussen et al., 2012; Knappe-Poindecker et al., 2013; Krull et al., 2014; Sullivan et al., 2015c; Zinicola et al., 2015b; Nielsen et al., 2016)
Facklamia (hominis)	Metagenomic sequencing	(Zinicola et al., 2015b)
Filifactor (villosus)	Metagenomic sequencing	(Zinicola et al., 2015b)
Fusobacterium (necrophorum)	Culture isolation, FISH, IHC, PCR, metagenomic sequencing	(Koniarová et al., 1993; Cruz et al., 2005; Berry et al., 2010; Rasmussen et al., 2012; Sullivan et al., 2015c; Nielsen et al., 2016)
*Guggenheimella (*bovis)	Culture isolation, qPCR, FISH	(Wyss et al., 2005; Strub et al., 2007; Schlafer et al., 2008)
Mycoplasma	Culture isolation, metagenomic sequencing, 16S rRNA sequencing	(Berry et al., 2010; Santos et al., 2012; Krull et al., 2014; Zinicola et al., 2015b; Nielsen et al., 2016)
Peptococcus	Culture isolation	(Koniarová et al., 1993)
Peptoniphilus (methioninivorax)	Metagenomic sequencing	(Zinicola et al., 2015b)
Peptostreptococcus	Culture isolation, 16S rRNA sequencing	(Koniarová et al., 1993; Berry et al., 2010; Santos et al., 2012)
Porphyromonas	Culture isolation, FISH, 16S rRNA gene sequencing, metagenomic sequencing	(Berry et al., 2010; Santos et al., 2012; Zinicola et al., 2015b; Nielsen et al., 2016)
Prevotella	Culture isolation	(Berry et al., 2010)
Propionispora (hippie)	Metagenomic sequencing	(Zinicola et al., 2015b)
Selenomonas (infelix)	Metagenomic sequencing	(Zinicola et al., 2015b)
Snowella (rosea)	Metagenomic sequencing	(Zinicola et al., 2015b)
Soehngenia (saccharolytica)	Metagenomic sequencing	(Zinicola et al., 2015b)

Staphylococcus	Culture isolation	(Berry et al., 2010)
Streptococcus	Culture isolation	(Koniarová et al., 1993; Berry et al., 2010)
Telmatospirillum (siberiense)	Metagenomic sequencing	(Zinicola et al., 2015b)
Treponema	Culture isolation, PCR, 16S rRNA gene sequencing, 16S-23S ISR rRNA gene sequencing, IHC, FISH, metagenomic sequencing	(Choi et al., 1997; Demirkan et al., 1998; Stamm et al., 2002; Cruz et al., 2005; Evans et al., 2008, 2009c, Klitgaard et al., 2008, 2013; Santos et al., 2012; Rasmussen et al., 2012; Krull et al., 2014; Zinicola et al., 2015b; Nielsen et al., 2016)

[a] *Species name is in parentheses where only one species in the genus has been identified*

[b] *Abbreviations: FISH, fluorescence in situ hybridisation; IHC, immunohistochemistry; qPCR, quantitative polymerase chain reaction.*

Despite the vast abundance of bacteria identified within DD lesions little is understood about how they contribute to DD lesion formation and progression. Only the *Treponema* genus belonging to the spirochaetes are consistently identified within DD lesions and appear to dominate the lesion microbiome thus suggesting treponemes are integral to DD aetiology (Krull et al., 2014; Zinicola et al., 2015b; Nielsen et al., 2016).

1.5.2 Treponemes associated with DD

A polytreponemal aetiology has come to light for DD following molecular, serological and isolation studies. Initially, five phylotypes of treponeme were identified in Germany through 16S rRNA gene sequencing of DD lesions (Choi et al., 1997). Since then a number of studies have also identified various treponemes of the same or different phylotypes in DD lesions (Stamm et al., 2002; Trott et al., 2003; Evans et al., 2008; Klitgaard et al., 2008; Nordhoff et al., 2008).

In the UK and USA three distinct cultivable phylogroups of DD-associated treponemes have been identified in DD lesions through both molecular and isolation methods (Stamm et al., 2002; Evans et al., 2008). These phylogroups were originally designated as the *Treponema medium / Treponema vincentii*-like phylogroup, the *Treponema phagedenis*-like phylogroup and the *Treponema putidum / Treponema denticola*-like phylogroup due to isolates sharing high 16S rRNA gene sequence identity (greater than 99% for the first two phylogroups and just over 95% for the latter phylogroup) with the human treponemes for which the phylogroups were named (Stamm et al., 2002; Evans et al., 2008). On the other hand, there was a greater difference in the 16S rRNA gene sequence identities between phylogroups; with the *T. phagedenis*-like DD treponeme phylogroup closest in sequence identity to the *T.*

putidum / T. denticola-like DD treponeme phylogroup with 92.3% whereas the *T. vincentii / T. medium*-like DD treponeme phylogroup was the most dissimilar to the *T. phagedenis*-like DD treponeme phylogroup with a shared 16S rRNA gene sequence identity of 90.1% (Evans et al., 2008).

Further genotype and phenotype characterisation of the *T. putidum / T. denticola*-like DD treponeme phylogroup lead to this phylogroup being designated as a new species of treponeme named *Treponema pedis* (Evans et al., 2009b). Furthermore, multi-locus sequence typing (MLST) of the three cultivable DD treponeme phylogroups demonstrated that there was very little diversity amongst isolates within each phylogroup which has lead to the removal of the '-like' suffix from the phylogroup names (Clegg et al., 2016b). Additionally upon analysis of the MLST data for the *T. vincentii / T. medium* DD treponeme phylogroup it was clear that *T. vincentii* did not belong in this phylogroup and thus the phylogroup was renamed the *T. medium* DD treponeme phylogroup (Clegg et al., 2016b).

Studies were able to demonstrate that DD treponemes co-populated DD lesions leading to a polytreponemal aetiology (Klitgaard et al., 2008; Schlafer et al., 2008; Evans et al., 2009c; Rasmussen et al., 2012). Evans et al., (2009c) demonstrated the polytreponemal nature of DD lesions using a PCR approach specific for each of the three cultivable DD treponeme phylogroups. Of the DD lesions investigated the *T. medium* DD treponeme phylogroup, *T. phagedenis* DD treponeme phylogroup and *T. pedis* DD treponeme phylogroup were detected in 96.1%, 98% and 76.5% respectively (Evans et al., 2009c). Additionally all three DD treponeme phylogroups were detected together in 74.5% of DD lesions (Evans et al., 2009c). Recently metagenomic studies have provided further evidence of a polytreponemal aetiology within DD lesions (Yano et al., 2010b; Santos et al., 2012; Klitgaard et al., 2013, 2014; Krull et al., 2014; Zinicola et al., 2015a; b; Nielsen et al., 2016). One study was able to show that treponemes undergo massive population shifts when the DD lesions progresses through the different stages of the disease, with a few phylotypes dominating each stage (Krull et al., 2014).

1.5.3 Experimental models of DD

To determine whether treponemes fulfil Koch's postulates for DD, a small number of studies have tried to induce DD experimentally. An initial study was able to induce DD lesion development in four-month-old calves by placing DD lesion homogenate on lightly abraded skin between the dew claws and the bulbs of the heel and keeping the foot continually wet and reducing oxygen concentration by using wrappings on the foot and leg

(Read and Walker, 1996). A later study was able to induce DD in one year old Holstein heifers using a similar method (without abrasion) and was also able to partially fulfil Koch's postulates by inducing a DD-like lesion in the region of the dew claw using a culture of a DD treponeme isolate (Gomez et al., 2012). More recently, a study systematically designed a protocol using macerated DD lesion material that enabled 95% success rate for DD induction (Krull et al., 2016). Similar to previous studies the skin barrier was compromised by abrasion and feet were kept moist through wrapping, however, moisture was maintained by wetting with sterile growth media (Krull et al., 2016). Although not the most successful protocol for DD induction, pure culture of *T. phagedenis* was able to induce DD lesions with a success rate of 59% (Krull et al., 2016). However, when *T. phagedenis* pure culture was combined with macerated lesion material and used as inoculum the induction rate of DD was less than that of the macerated lesion material alone (Krull et al., 2016). Thus whilst DD treponemes are able to initiate DD lesions, it would appear that other factors present in lesion material may be required for optimum DD lesion induction.

1.6 The *Treponema* genus

1.6.1 *Treponema* biology

The *Treponema* genus belongs to the Spirochaetes, a phylum of bacteria containing a number of genera important to human and animal health. Treponemes are host associated, fastidious, anaerobic (or microaerophillic), spiral-shaped (Figure 1.2 and 1.3), gram negative microorganisms. As a member of the spirochaetes, *Treponema* have a unique ultrastructure of a cytoplasmic cyclinder composed of the nuclear region and cytoplasm enclosed by the cell membrane and cell wall which is surrounded by an outer sheath (Figure 1.2) (Canale-Parola, 1977; Brenner et al., 1984; Radolf and Lukehart, 2006). Depending upon species, treponemal cells are typically 5-20 μm in length and 0.1-0.5 μm in diameter with tight spirals that are either regular or irregular and vary in number (Brenner et al., 1984; Radolf and Lukehart, 2006). Spirochaetes, including treponemes, have the ability to change to a spherical shape (known as either the encysted form, round body or spherical body) (Figure 1.3) in response to stresses in the environment such as stage of culture and then revert back to the spiral form when conditions are favourable (Brorson and Brorson, 1997; Murgia and Cinco, 2004; Döpfer et al., 2012a).

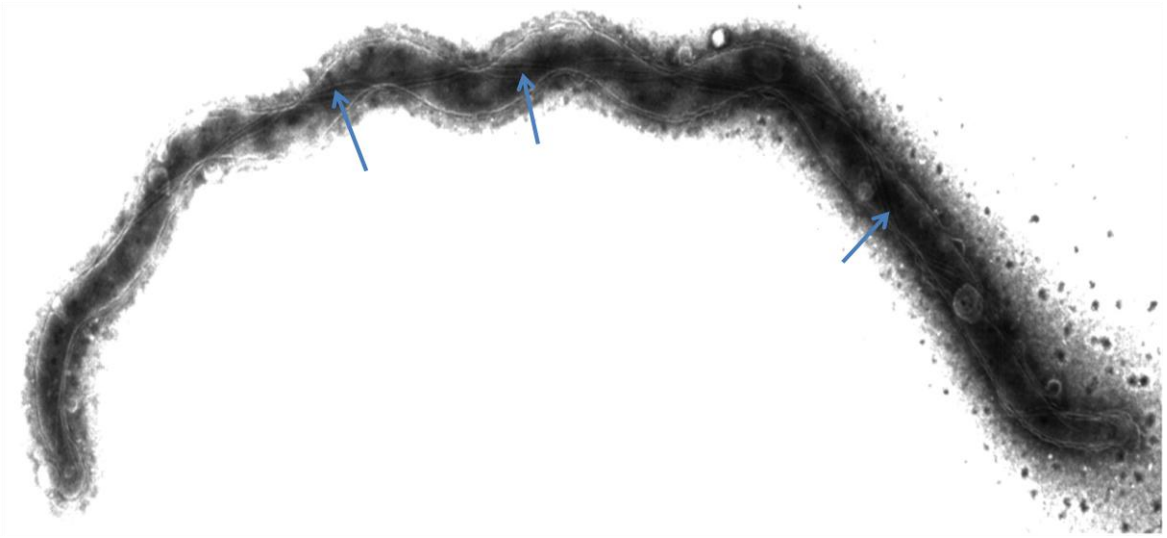


Figure 1.2: Electron micrograph of a DD treponeme

Electron micrograph of T. pedis DD treponeme phylogroup strain T3552B negatively stained cells providing an example of the typical spiral morphology of DD treponemes(Evans et al., 2009b). *Arrows point to the endoflagella that are only just visible spanning the periplasmic space between the cell wall and the outersheath. T3552B has three endoflagella spanning from each polar end which overlap in the middle. Figure adapted from* Evans et al., (2009b).

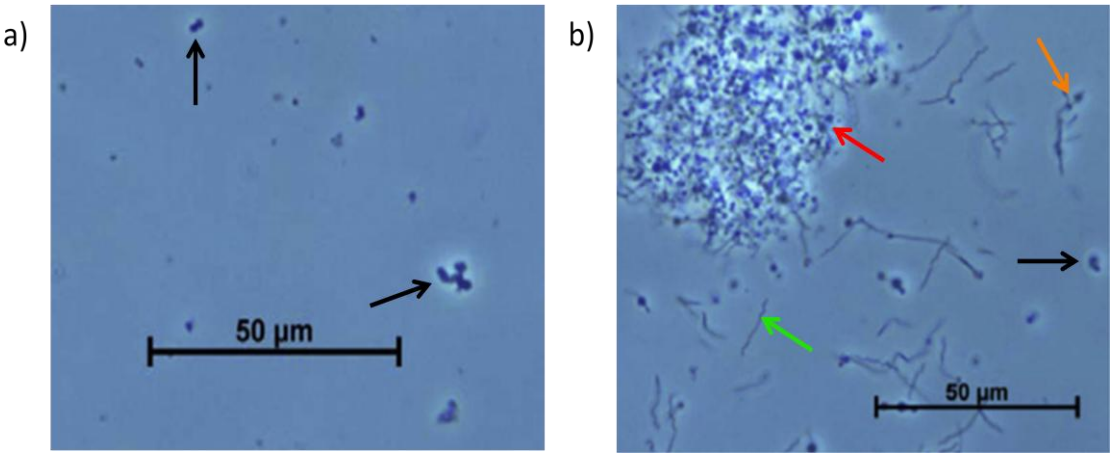


Figure 1.3: The encysted form of treponemes

Phase contrast microscopy images of a) a day 0 culture of a T. phagedenis isolate only present in the encysted form (arrows point to examples of the encysted forms) (Döpfer et al., 2012a). b) A day 9 culture of a T. phagedenis isolate in which multiple morphological forms are present. The black arrow points to an example of the encysted form, the green arrow points to an example of the spiral form (characteristic morphology associated with treponemes), the orange arrow points to an intermediate morphology which shows the spiral form with a spherical body at the tip and the red arrow points to the clumping of encysted forms/ spherical bodies with the spiral form visible at the periphery of the clump (Döpfer et al., 2012a). Scale bars are shown for 50 µm. Figure is adapted from Döpfer et al., (2012a).

The rotational, translational and jerky motility of treponemes is imparted by the presence of endoflagellum unique to spirochaetes (Figure 1.2). The flagella arise from each polar end of the cell and span the periplasmic space between the cell wall and the outersheath where they may overlap with each other in the middle (Canale-Parola, 1978; Charon and Goldstein, 2002) Typically a treponeme may contain between one and eight flagella per cell (Radolf and Lukehart, 2006). The motility of treponemes is considered an important mechanism for *Treponema* virulence, especially as it allows the treponemes to move through viscous environments which may be found in the host or environment.

1.6.2 Pathogenic treponemes important in medical and veterinary health

Treponemes may be pathogenic to humans and livestock. Perhaps the most well studied pathogenic treponeme is *Treponema pallidum* subsp. *pallidum*, the causative agent of syphilis (Noordhoek et al., 1990). The disease is sexually transmitted and results in a systemic infection which initially causes localised development of ulcerative lesions (chancre) in the early stages of the disease (Singh and Romanowski, 1999; Radolf and Lukehart, 2006). As the disease progresses, other parts of the body become affected and it can eventually lead to neurological and cardiac problems (Singh and Romanowski, 1999; Radolf and Lukehart, 2006). Additionally syphilis is known to facilitate HIV co-infection as well as impact upon HIV pathogenesis (Kofoed et al., 2006). Another subspecies of *T. pallidum* is the causative agent of Yaws, an infection of the skin, bone and cartilage, which unlike syphilis is not a sexually transmitted disease and is transmitted through skin to skin contact (Noordhoek et al., 1990).

Treponemes are part of the polymicrobial complex that cause periodontal diseases in humans and animals which results in chronic inflammation of the gingival tissue, connective tissue and bone in the oral cavity (You et al., 2013). A range of treponemes have been detected in periodontal diseases including *T. denticola, Treponema pectinovorum and T. vincentii,* with *T. denticola* strongly implicated in disease progression (Choi et al., 1994; Edwards et al., 2003b; You et al., 2013; Borsanelli et al., 2015, 2017). Periodontal disease is often paralleled with DD as both disease are characterised by chronic inflammatory tissue destruction as a result of a poorly understood polymicrobial complex comprising of a number of treponemal species (Choi et al., 1997; Edwards et al., 2003a; b).

Virulence mechanisms of treponemes are still being deciphered. However, some treponemes possess certain virulence factors which may enable them to be pathogenic under the right circumstances. Briefly, oral treponemes have been shown to have haemolytic activity and β-haemolysis has been demonstrated in two phylogroups of DD treponemes (Chu et al., 1994; Evans et al., 2008). *T. denticola* has the ability to co-aggregate with other oral bacteria, which may enable colonisation and biofilm formation (Yao et al., 1996; Rosen et al., 2008; Dashper et al., 2011). Additionally, treponemes have also been shown to have a range of protease activities which can contribute to virulence (Ohta et al., 1986; Miyamoto et al., 2006; Evans et al., 2008).

1.6.3 Non-pathogenic commensal treponemes

There is a large array of non-pathogenic treponemes which are commensal in humans and animals. The gastrointestinal (GI) tract of ruminants plays host to a wide variety of non-pathogenic symbiotic treponemes including *Treponema byranttii* and *Treponemea sacchrophilum* (Stanton and Canale-Parola, 1980; Paster and Canale-Parola, 1985). A recent study characterised seven isolates from the GI tract which were novel in morphology and genetically different to previously characterized GI treponemes (Evans et al., 2011b). Indeed, two of these isolates were recently named as new species *Treponema rectale* and *Treponema ruminis* (Evans et al., 2011b; Newbrook et al., 2017; Staton et al., 2017). It was also determined that these GI treponemes were both genetically and phenotypically distinct from treponemes associated with DD (Evans et al., 2011b). Other examples of commensal treponemes include *T. phagedenis* in the genitalia of humans (although appears to have a pathogenic role in DD), *Treponema primitia* and *T. azotonutricum* in the hindgut of termites (Graber et al., 2004).

1.6.4 Detection methods for treponemes

The fastidious anaerobic nature of treponemes means that they are difficult to cultivate and isolate. Indeed, treponemes involved in the human diseases of syphilis, yaws and pinta cannot be cultivated and require a live host for growth (Radolf et al., 2016). There has been success with culture for treponeme detection with regards to non-pathogenic GI treponemes and pathogenic treponemes involved in periodontal disease and DD (Choi et al., 1994; Chu et al., 1994; Walker et al., 1995; Stamm et al., 2002; Trott et al., 2003; Evans et al., 2008; Sullivan et al., 2013, 2014b, 2015b; Clegg et al., 2015). However, many treponemes remain uncultivated with an estimate of 75% of oral treponemes having not been cultured (Choi et al., 1994; Dewhirst et al., 2000). Although cultivation of these

treponemes is still not without its difficulties, especially as the presence of the encysted morphological form of treponemes in cultures (Section 1.6.1) may result in false negatives if only the spiral form is considered for identification (Döpfer et al., 2012a). The difficulties in culturing DD treponemes have meant that culture techniques for detection such as phage typing, in which susceptibility of bacterial colonies to specific bacteriophages are used as means of bacterial identification, cannot be readily applied to DD treponeme detection. Additionally little is known about the bacteriophages for treponemes (Szafrański et al., 2017).

Treponemes have been successfully detected by molecular and serological methods which can overcome the inability of some treponemes to be cultivated. The techniques include PCR utilising the 16S rRNA gene which may also be used for sequencing (Choi et al., 1997; Dewhirst et al., 2000; Stamm et al., 2002; Klitgaard et al., 2008; Evans et al., 2009a), fluorescence *in situ* hybridisation (FISH) (Klitgaard et al., 2008; Rasmussen et al., 2012), immunohistochemistry (Cruz et al., 2005; Evans et al., 2009c; Angell et al., 2015b), immunocytochemsitry (Demirkan et al., 1998), ELISA (Demirkan et al., 1999; Murray et al., 2002; Dhawi et al., 2005), DNA-DNA dot blot (Nordhoff et al., 2008) and metagenomics (Klitgaard et al., 2013, 2014; Krull et al., 2014; Gao et al., 2015; Zinicola et al., 2015a; Nielsen et al., 2016).

Whilst ELISA provides useful information about whether cattle have been exposed to DD treponemes and their reactivity to particular treponemal phylogroups and antigens (Demirkan et al., 1999; Murray et al., 2002; Dhawi et al., 2005; Gomez et al., 2014a), its application as a diagnostic tool for DD or active infection with DD treponemes is problematic. Such immunological detection is limited due to the endemic nature of DD resulting in nearly all cattle from an infected herd being seropositive to DD treponemes to some extent (Orsel et al., 2017). In addition this method cannot be used to determine where infection may be occurring within the animal. Staining and immunohistochemical methods have been useful for determining the localisation of DD treponemes within tissues (Cruz et al., 2005; Evans et al., 2009a; Angell et al., 2015b), however, many of these methods do not differentiate between DD treponeme phylogroups, have low specificity and sensitivity and the process can be expensive and time consuming.

Molecular methods have become the mainstay of DD treponeme detection. Particularly PCR based methods which enable large numbers of samples to be processed at low cost to quickly determine DD treponeme presence/ absence in lesions directly from the tissue or

from cultures which is very useful for epidemiological and exploratory studies. In addition, DD treponemes can be detected to the species or phylogroup level using either specific PCR assays or the sequencing of isolate or tissue derived PCR amplicons (Choi et al., 1997; Dewhirst et al., 2000; Stamm et al., 2002; Evans et al., 2008, 2009c).

To acquire more information about DD treponemes other than presence/ absence additional molecular techniques need to be utilised. Metagenomic studies, often involving deep sequencing, have enabled a plethora of treponemes to be detected within diseased tissues and enabled their general abundance in the microbiome to be determined (Klitgaard et al., 2013, 2014; Krull et al., 2014; Gao et al., 2015; Zinicola et al., 2015a; Nielsen et al., 2016). Indeed, deep sequencing methods are advantageous over conventional PCR methods as this more sensitive method enables microorganism that comprise < 1% of the microbiome of the sample of interest to be detected (Klitgaard et al., 2014), whereas conventional PCR methods are not this sensitive. However, a limitation of deep sequencing for DD treponeme detection is that although next generation sequencing is becoming more cost efficient it is still more expensive than other methods and it requires much more labour intensive data analysis which makes it predominantly unsuitable for larger epidemiological studies or application as a general diagnostic tool.

More recently real time PCR assays have been developed for the detection of the three cultivable DD treponeme phylogroups (*T. medium* phylogroup*, T. phagedenis* phylogroup and *T. pedis* phylogroup) (Anklam et al., 2017). Such assays offer a better alternative to conventional PCRs as real time PCRs are more sensitive due to real time detection during the exponential phase of amplification. Furthermore a large number of samples can be processed and pathogen abundance can be qualitatively or quantitatively determined. Loop-mediated isothermal amplification (LAMP) assays have also been developed for the *T. medium, T. phagedenis* and *T. pedis* DD treponeme phylogroups which is also more sensitive and specific than conventional PCRs and proven to be equally as sensitive and specific as the real time PCR assays, requires less labour, is fast and does not require a thermocycler (Notomi et al., 2000; Anklam et al., 2017). Although a disadvantage is that LAMP is more suited to presence/ absence studies as quantification requires additional machinery which off sets the advantage of this method being low cost (Anklam et al., 2017).

A completely different method which has yet to be applied to DD lesions is matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS). Microbial

identification is possible by comparing the peptide mass fingerprint obtained for an isolate of a microorganism to a microbial mass spectra database (Bizzini and Greub, 2010; Singhal et al., 2015). MALDI-TOF MS is advantageous in that it is very rapid (within minutes), reported to have very good accuracy, reagents are inexpensive, samples require minimal preparation and it can detect taxa to either the genera, species, subspecies or strain (Bizzini and Greub, 2010; Singhal et al., 2015). However, initial set up of MALDI-TOF can be expensive and the majority of work with MALDI-TOF MS is with culture isolates (Calderaro et al., 2013; Singhal et al., 2015), which would severely limit its application to the fastidious DD treponemes.

Whilst the aforementioned techniques enable detection of DD treponemes and enhance understanding of their prevalence and abundance within DD lesions, bacterial cultivation continues to be a requisite to clarify phenotypic diversity and dissect the aetiopathogenesis of this important disease.

1.7 The promiscuous nature of DD treponemes

1.7.1 Manifestations of DD in animals other than dairy cattle

In recent years DD-like manifestations have emerged in animals other than dairy cattle. Although, less common than in dairy cattle DD has now been confirmed in beef cattle (Sullivan et al., 2013). In sheep, DD is known as contagious ovine DD (CODD) and is a much more severe form of DD than seen in cattle. It was originally described in 1997 as a severe virulent ovine foot root but the detection of DD treponemes within the lesions has lead to its classification as a form of DD (Harwood et al., 1997; Davies et al., 1999; Dhawi et al., 2005; Moore et al., 2005; Sayers et al., 2009). Recently, infections manifesting in a similar manner to CODD have been identified in dairy goats and Wild North American Elk (Sullivan et al., 2014b; Clegg et al., 2015). Whilst not infecting the foot, DD treponemes have also been identified in porcine ear necrosis, shoulder ulcers, tail lesions and flank lesions (Pringle et al., 2009; Svartström et al., 2013; Karlsson et al., 2014; Clegg et al., 2016e).

1.7.2 Detection of DD treponemes in other types of dairy cattle infections

Investigations into other infections and destructive tissue problems experienced by dairy cattle has lead to the discovery that DD treponemes are not solely associated with DD lesions. Three types of claw disorder known as sole ulcers, toe necrosis and white line disease are typically not caused by infection. However, DD treponemes may be detected in these disorders if they become chronic, impervious to normal treatments (‘non-healing’),

appear granulomatous and smell repugnant (similar to DD) (Evans et al., 2011a). Additionally in the skin of limbs from dairy cattle DD treponemes have been detected in bovine pressure sores and open hock lesions (including the skin surrounding the lesion) (Clegg et al., 2016a; d). DD treponemes have also been identified in infections involving the udders known as ulcerative mammary dermatitis and more recently the teats in ischaemic teat necrosis (Evans et al., 2010; Clegg et al., 2016c), which can result in total loss of the teat and thus premature culling of cattle (Clegg et al., 2016c). Although DD treponemes appear to be highly associated in many of these infections, they are only 100% associated with bovine pressure sores and open hock lesions (Clegg et al., 2016a; d), suggesting for the other infections that their role in pathogenesis of these polymicrobial infections may not be as pivotal.

1.8 Diagnosis, treatment and control of DD

1.8.1 Diagnosis of DD on farm

Diagnosis of DD is definitively given if a DD lesion is present on the foot upon inspection. Lameness and other signs of discomfort (for example, shifting of weight from one foot to the other) can be indicative of DD and would typically result in further investigation of the cause which would identify DD if present (Bassett et al., 1990). However, lameness does not always occur with DD and thus the feet may only be inspected during routine lifting of the feet; for example, during routine foot trimming, which would result in episodes of DD being missed. Lifting feet frequently for the purpose of screening for DD, especially in large herds, would be labour intensive, time consuming and an added stress for the cow, and is therefore not particularly feasible (Rodriguez-Lainz et al., 1998; Relun et al., 2011).

Studies have investigated the effectiveness and reliability of screening for DD in the milking parlour to try and negate issues with lifting the feet for inspection, particularly for research purposes although could also be applied to farm management practice (Rodriguez-Lainz et al., 1998; Thomsen et al., 2008; Relun et al., 2011; Stokes et al., 2012a; Solano et al., 2017). Sensitivity and specificity compared to lifting feet for inspection were relatively good depending upon the study with sensitivity ranging between 0.65 and 1.00 and specificity ranging between 0.80 and 0.99 for different studies (Rodriguez-Lainz et al., 1998; Relun et al., 2011; Solano et al., 2017). Variation in sensitivity and specificity described could be due to differing experimental designs including scoring method, parlour configuration and observation time length (Stokes et al., 2012a). The sensitivity and

specificity values for screening for DD lesions in the parlour described earlier were based upon presence/ absence of DD lesions, however, it was also found that sensitivities and specificities of parlour DD lesion screening varied between different lesion stages (Stokes et al., 2012a; Solano et al., 2017). Thus with routine parlour screening cows that are not lame from DD may be picked up by this method and treated , however, this method does add time to the milking, requires washing of the feet which is added labour to the milking process and risks teat infection from splash back and is not sufficient for M-stage lesion progression follow up (Rodriguez-Lainz et al., 1998; Relun et al., 2011; Solano et al., 2017).

1.8.2 Treatment and control of DD

Cattle may either be treated for DD at the individual level with topical sprays or at the herd level with footbaths (Laven and Logue, 2006). The use of antibiotics such as oxytetracycline topical sprays and erythromycin footbaths have been shown to reduce DD on farm (Blowey and Sharp, 1988; van Amstel et al., 1995; Laven and Proven, 2000; Cruz et al., 2001). However, recent studies looking at the antimicrobial susceptibilities of treponemes associated with DD show that they only have an intermediate susceptibility to the most commonly used antibiotics including oxytetracycline, spectinomycin and lincomycin, and that antibiotics in footbaths are not used at efficacious concentrations for use against treponemes (Evans et al., 2009a, 2012a; Yano et al., 2010a). Whilst some healing is grossly apparent after use of these intermediate susceptibility antibiotics, probably as a result of removing other bacterial genera within the lesion, it is highly likely that they are not effectively removing the treponemes which are considered the primary agents of DD (Evans et al., 2016). DD treponemes are able to convert to an encysted morphology (see Section 1.6.1) which has been associated with a lack of antibiotic efficacy in the spirochaete *Borrelia burgdorferi* (Murgia and Cinco, 2004; Döpfer et al., 2012a). Additionally, poor tissue penetration of topical treatments is an issue and as DD treponemes are often found deep within lesions the topical treatments may also not be reaching them (Klitgaard et al., 2008; Evans et al., 2009c, 2016; Rasmussen et al., 2012). Thus, this possible lack of bacteriological cure may explain the reactivation of M4 lesions to M4.1 following treatment as well as the larger amount of lesion reoccurrence that is observed in follow up after treatment and the observation of spirochaetes in clinically healed lesions (Berry et al., 2010, 2012).

Systemic antibiotics are an alternative to topical antibiotics; however, there are a limited number of studies investigating the efficacy of systemic antibiotics against DD in cattle.

Trials have been conducted for the investigation of systemic use of aqueous procaine penicillin G, ceftiofur sodium, cefquinome and erythromycin as a treatment for DD (Read and Walker, 1998; Laven, 2006). These systemic antibiotic treatment studies have had varying results with in some cases only a reduction in lesion severity recorded and reoccurrence of DD in treated cattle observed (Read and Walker, 1998; Laven, 2006). Additionally, the minimum inhibitory concentration (MIC) for ceftiofur against DD-associated treponemes demonstrates an intermediate susceptibility which reduces the likelihood of bacteriological cure, especially if the dosage is not appropriate (Evans et al., 2012a). Whilst the *in vitro* susceptibility of cefquinome for treponemes is unknown, penicillin and erythromycin have been shown to have extremely low MICs for DD treponemes and these antibiotics have been highly effective as treatment for human treponemal infections (Fernando, 1969; Idsoe et al., 1972; Evans et al., 2009a, 2012a; Angell et al., 2015a). However, to be effective in humans these antibiotics are often given over a much longer duration than what has been investigated in cattle (Evans et al., 2016). Additional considerations for actual use of these (and other) antibiotics that DD treponemes have high susceptibility to (e.g. amoxicillin) are that licensing for use in cattle for DD may be difficult as the antibiotics are considered critical for human health and for some instances use may require milk withdrawal which can be economically costly (Laven and Logue, 2006; WHO, 2011; Evans et al., 2016).

More general concerns regarding antibiotic use to treat DD include increasingly strict legislations limiting use and antimicrobial resistance being acquired both by treponemes (Evans et al., 2009a) and potentially other host associated microbiota during treatment. Taken together, given the poor bacteriological cure of topical treatments and the implementation difficulties involved with systemic antibiotics there are a number of barriers to antibiotics being an effective method for DD control in dairy herds.

Non-antimicrobial options are available for use as topical treatments and for footbaths, which have been shown to reduce DD prevalence in some cases (Hernandez et al., 1999; Laven and Logue, 2006; Speijers et al., 2010). Commonly, these alternatives involve formalin/ formaldehyde or copper sulphate which are currently undergoing upheavals in legislation of use due to the former being reclassified as a Category 1B carcinogen in the Europe Union (Commission Regulation (EU) 605/2014) (Salthammer, 2015) and the latter being environmentally damaging (Laven and Logue, 2006; Evans et al., 2016). A problem with many of the alternatives used to replace antibiotics is that their regimens can be very

laborious and still do not sufficiently control DD on farm (Britt et al., 1996; Laven and Logue, 2006).

Whilst the varied current treatment strategies are able in most cases to reduce DD prevalence following initial treatment, not all cows treated benefit and reoccurrence is high which is possibly due to reactivation of the lesion due to suboptimal treatment for treponemes or re-infection from another cow. Therefore new control strategies focussing on prevention as opposed to being reactive may provide a better means of control and enable progression towards elimination of DD on farms.

Recent studies have investigated some novel preventative measures for control. One study investigated the role of nutrition in prevention and evaluated the use of an organic trace mineral and iodine premix in its ability to enhance resistance to DD development upon natural exposure and experimentally induced exposure (Gomez et al., 2014b). Whilst initial results from experimentally DD induced animals looked promising for a reduction in DD induction and severity following use of the premix compared to the control, further work is required to validate this effect (Gomez et al., 2014b). Another study investigating preventative measures in terms of hygiene found that automatic washing of cattle feet during milk reduced the prevalence of DD in the washed feet (Thomsen et al., 2012). More targeted prevention strategies can be designed based on risk factors for DD as well as through the identification of DD infection reservoirs.

1.9 Risk factors for DD

The identification of risk factors can help farmers to make informed decisions about how to best reduce the risk of DD on farm and can inform upon potential DD infection reservoirs. Risk factors may be split into animal level risk factors and management and environment risk factors.

1.9.1 Animal level risk factors

Certain risk factors may only affect a sub-group of animals within a herd which may result in them being at a higher risk for DD infection. These risk factors have been summarised in Table 1.2. Breed of cow has been cited as a risk factor for DD with pure and cross breeds of Holstein-Friesians deemed to have higher susceptibility to DD than other breeds (Rodriguez-Lainz et al., 1999; Holzhauer et al., 2006; Relun et al., 2013a). Indeed, Rodriguez-Lainz et al., (1999) found that cows that were the dual purpose German Red-

Pied breed were significantly less at risk of DD than the German Black-Pied x Holstein breed. Holzhauer et al., (2006) similarly found that the dual purpose Meuse-Rhine-Issel (MRIJ) breed had a lower risk of DD than Holstein-Friesian breed. Again, Relun et al.,(2013a) found Holsteins to be more at risk of DD than other breeds used in France. Another study investigating lameness as opposed to specific foot lesions also found that dairy farms using breeds other than the Holstein-Friesian were less likely to be lame (Barker et al., 2010). This data is highly suggestive that certain breeds may have a genetic factor that infers a level of susceptibility to DD.

Table 1.2: Risk factors for DD at the animal level

Animal level risk factor	Effect on DD prevalence	References
Breed	• Holstein –Friesians and the associated cross breeds have a higher risk of DD • Lower risk breeds include German Red-Pied, MRIJ (dual purpose breed) and Normande (and other French breeds)	Rodriguez-Lainz et al., 1999; Holzhauer et al., 2006; Relun et al., 2013
Parity	• First parity at higher risk • Risk reduces with each parity following the first (or second depending on study)	Read and Walker, 1998; Rodriguez-Lainz et al., 1999; Somers et al., 2005; Vink, 2006; Barker et al., 2009
Lactation	• Increased risk during lactation compared to the dry period • Risk is even higher during mid-late lactation • High yielding cows are at greater risk than low yielding • Increased risk in the first month after calving	Argáez-Rodríguez et al., 1997; Read and Walker, 1998; Murray et al., 2002; Somers et al., 2005; Holzhauer et al., 2006; Barker et al., 2009; Relun et al., 2013

The parity of the animal has also been linked as a risk factor for DD. Studies have found first-parity cows were more at risk of DD than multiparous and risk reduces with each parity (Rodriguez-Lainz et al., 1999; Somers et al., 2005; Holzhauer et al., 2006; Barker et al., 2009). Some studies cite second parity as least at risk of DD but this may be due to different management practices with first-parity cows (Argáez-Rodríguez et al., 1997; Holzhauer et al., 2006; Vink, 2006). It is considered that risk of DD is higher in first-parity cows due to the stress of first calving as a result of metabolic and environmental changes associated with this experience (Somers et al., 2005).

Lactation is cited as a risk factor for DD with studies noting an increased risk of DD in lactating cows than in dry cows (Read and Walker, 1998; Murray et al., 2002; Somers et al., 2005; Holzhauer et al., 2006; Relun et al., 2013a). It has been postulated that this trend is due to the diet of lactating cows compared to dry cows in which the latter is fed more roughage resulting in firmer faeces as opposed to the liquid faeces of lactating cows (Somers et al., 2005). Therefore dry cow housing is drier due to less slurry and thus more hygienic (Somers et al., 2005). There are contrasting results for the period of lactation in which DD is more of a risk. One study found the risk was highest during peak lactation and another found an increased risk within the first month of calving (Argáez-Rodríguez et al., 1997; Holzhauer et al., 2006).

1.9.2 Farm level risk factors

Farm level risk factors typically affect the majority of the animals on the farm and are associated with the environment and farm management practices. Risk factors for DD at farm level are listed in Table 1.3. A number of risk factors have been identified with herd management practices. Increasing herd size has been cited as having an increased risk for DD which is likely due to increased crowding and associated hygiene (Rodríguez-Lainz et al., 1996; Rodriguez-Lainz et al., 1999; Wells et al., 1999; Oliveira et al., 2017). Buying-in replacement heifers is a major risk factor for increased risk of DD on farm (Rodríguez-Lainz et al., 1996; Wells et al., 1999; Oliveira et al., 2017). The infectious nature of DD means that if cattle are bought in with DD or a history of DD then the disease will quickly spread to the rest of the herd from the current lesion or even through potential reactivation of an old lesion (Read and Walker, 1998; Berry et al., 2012), thus presenting a significant biosecurity risk. Furthermore cattle new to the herd may be stressed due to the new environment which may make them vulnerable to DD already on farm (Read and Walker, 1998). Additional management practices which increase the risk of DD include introducing dry cows to the lactating herd before calving occurs as opposed to after calving (Somers et al., 2005) and when calving season is during the winter months (Rodriguez-Lainz et al., 1999). How often cattle are trimmed is another management practice which affects the risk of DD. Somers et al., (2005) found that there was an increased risk of DD if there was greater than 7 months between foot trimming for each cow. However, other studies have reported increased risk of DD with shorter durations between hoof trimming (Wells et al., 1999; Holzhauer et al., 2006).

Table 1.3: DD infection reservoirs at the farm level

Farm level risk factor	Effect on DD prevalence	References
Biosecurity	• Increased risk with muddiness • Reduced risk with increased animal hygiene • Increased risk with no cleaning/ disinfection of foot trimming equipment between cows • Increased risk with using a professional foot trimmer who trims on other farms • Farms that use footbaths are at lower risk than farms who do not • Increased risk with reduced manure scraping frequency • Increased risk if water troughs are contaminated with manure • Increased risk if manure scraping vehicle was used for other purposes • Increased risk with no boots available for visitors when compared to farms with boots available • Increased risk if farm staff worked with other herds • Increased risk if trucks collecting animals for slaughter have access to barn	Rodríguez-Lainz et al., 1996; Rodriguez-Lainz et al., 1999; Wells et al., 1999; Hultgren and Bergsten, 2001; Somers et al., Relun, 2013, 2005; Oliveira et al., 2017
Housing	• Reduced risk with keeping cows on pasture compared to housed • Daily winter pasture access reduces risk compared to permanently housed • Reduced risk with straw yards compared to other housing systems • Reduced risk with thicker bedding	Blowey and Sharp, 1988; Laven, 1999; Rodriguez-Lainz et al., 1999; Wells et al., 1999; Somer et al., 2003; Somers et al., 2005; Onyiro et al., 2008; Barker et al., 2009
Floor system	• Increased risk with grooved concrete > smooth/ slatted concrete > textured concrete • Increased risk with solid concrete floor > slatted floor > slatted floor with scraper • Solid concrete floor had an increased risk compared to rubber	Wells et al., 1999; Hultgren and Bergsten, 2001; Somers et al., 2005; Barker et al., 2009
Nutrition	• Feeding maximum concentrate levels two weeks after calving increased the risk of DD compared to feeding maximum levels three or more weeks later • Increased risk with feeding by-products • Increased risk if cows body score is too high or too low • Possible reduced risk of DD when fed higher than the recommended amounts of organic trace minerals and iodine	Somers et al., 2005; Schöpke et al., 2013; Gomez et al., 2014
Herd and other	• Reduced risk with foot trimming twice per year with risk increasing the longer the period between trims • Increased risk with larger herd size • Introduction of a dry cow into a lactating herd before calving increases risk • Increased risk when calving season is in winter • Increased risk with buying in replacement heifers	Rodríguez-Lainz et al., 1996; Rodriguez-Lainz et al., 1999; Wells et al., 1999; Somers et al., 2005; Oliveira et al., 2017

The level of hygiene and cleanliness on a farm can have a significant impact on DD prevalence, with an increased risk of DD-associated with muddiness of corals (Rodríguez-Lainz et al., 1996), poor animal hygiene (Hultgren and Bergsten, 2001), low frequency of manure scraping and water troughs contaminated with manure (Oliveira et al., 2017). The high levels of manure, slurry and mud in the farm environment will affect the cleanliness of the cow. Contact of slurry with the skin can result in maceration which is necessary for DD development in animal experiment models (Gomez et al., 2012; Krull et al., 2016). Maceration of bovine skin by slurry has been shown in a small study to increase the skins permeability and thus may facilitate infection with DD-associated treponemes (Palmer et al., 2013).

The following biosecurity risks have been highlighted as factors for the increased risk of DD on a farm. The use of professional foot trimmers and farm workers who work on multiple operations are associated with an increased risk of DD on farm (Wells et al., 1999; Oliveira et al., 2017). Additionally in terms of spread between operations, an increased risk of DD has been associated with: access of the truck for slaughter animals to housing areas, no provision of boots for visitors and use of the manure scraping vehicle for purposes other than scraping (Oliveira et al., 2017). Furthermore, there is an increased risk of DD when foot trimmers do not wash or disinfect there tools between animals (Wells et al., 1999). Therefore the risk of DD would seem connected to poor biosecurity.

Risk factors associated with housing can also have an effect on DD. Several studies have found that increased access to pasture results in a decreased risk in DD (Blowey and Sharp, 1988; Rodríguez-Lainz et al., 1996; Read and Walker, 1998; Wells et al., 1999; Somers et al., 2005; Onyiro et al., 2008). The housing type has also been implicated as a factor for DD risk with cattle housed in straw yards typically at a reduced risk of DD compared to those housed in cubicles (Laven, 2001; Onyiro et al., 2008), although one study showed that after 6 months housed, straw yards had a similar risk to cubicles (Onyiro et al., 2008).

Flooring system used in housing also had an effect on DD prevalence and is therefore considered a risk factor. Grooved flooring has been shown to be high risk for DD compared to textured concrete with smooth or slatted concrete having an intermediate effect between the two (Wells et al., 1999). A different study found that a solid concrete floor was high risk for DD compared to a slatted floor with a scraper system and a slatted floor without a scraping system had an intermediary effect between the other two (Somers et al., 2005). Additionally rubber floors reduce the risk of DD when compared to solid

concrete floors (Hultgren and Bergsten, 2001). The association of DD with floor type is likely to be both for hygiene and mechanical reasons. The use of certain floors, such as slatted floors with scraping systems, enables better drainage and removal of slurry (Somers et al., 2005; Barker et al., 2010). On the other hand, grooved concrete floors can retain slurry following scraping (Palmer and O'Connell, 2015). Furthermore concrete can be extremely slippery and abrasive depending upon the type used and its maintenance (Wells et al., 1999; Barker et al., 2010), which can cause damage to the barrier defence of the skin of the foot which may enable pathogen entry. Indeed, abrasion in addition to maceration is another pretreatment requirement for DD induction in experimental animals (Krull et al., 2016).

Finally nutrition also plays a role in risk of DD in addition to the aforementioned role in faecal consistency. When the maximum level of concentrate during stepping up of concentrate rations was given less than two weeks following calving there was an increased risk of DD compared to those that received the maximum level of concentrate greater than two weeks post calving (Somers et al., 2005). This may have lead to a metabolic imbalance increasing susceptibility to DD (Enevoldsen et al., 1994; Somers et al., 2005). In addition, the feeding of by-products rich in protein from the food industry was associated with an increased risk for DD (Somers et al., 2005), which may be due to an excessive protein intake for the cows needs which has previously been described as a DD risk factor (Bargai, 1994; Somers et al., 2005). A non-optimum body condition score (too high or too low) has been associated with DD, which has been postulated to be due to poor nutrition leading to metabolic imbalances resulting in stress which weakens the immune system (Schöpke et al., 2013). A study also found that cattle fed a premix containing higher than the recommended amounts of organic trace minerals and iodine developed less DD lesions compared to a control group fed a standard trace mineral premix when the cattle were experimentally challenged to induce DD lesion development (Gomez et al., 2014b). Indeed it was found there was reduced risk (RR = 0.54, P = 0.11) of M2 stage development, that was not statistically significant, in cattle fed the high organic trace mineral and iodine premix (Gomez et al., 2014b). Further work is required to substantiate whether feeding higher than the recommended amount of organic trace minerals and iodine leads to a reduced risk of DD development.

1.10 Infection reservoirs of DD-associated treponemes

How DD is spread on and between farms is currently under investigation. Risk factor studies, although contradictory at times, clearly suggest that hygiene and biosecurity practices are important for DD infection. Additionally, the high association of treponemes in DD lesions of cattle suggests that DD treponemes can colonise and invade the feet of cattle and thus the conditions within cattle feet at least, must be survivable for DD-associated treponemes. The identification of infection reservoirs of DD treponemes is an important step in understanding how transmission of DD is occurring. An infection reservoir with regards to DD treponemes refers to an environment other than the DD lesion itself where DD treponemes can survive and/ or multiply and thus enables their subsequent transmission. Studies have begun trying to decipher where infection reservoirs may be and a summary of the current findings are in Table 1.3.

1.10.1 Infection reservoirs within dairy cattle tissues.

Evans et al., (2012b) carried out a whole dairy cow survey on six cattle, as well as investigating additional samples of the tissues of interest, to determine where in the cattle, other than the DD lesion themselves, the three DD treponeme phylogroups (*T. medium, T. phagedenis* and *T. pedis*) may be present (Table 1.4). The study detected one or more of the three DD treponeme phylogroups by PCR in the gingiva of the oral cavity and rectum of 14.3% and 14.8% of cattle investigated (Evans et al., 2012b). DD treponeme positive gingiva samples were only detected in DD-affected cattle whereas the DD treponemes in the rectum were detected in both DD-affected and unaffected cattle (Evans et al., 2012b). Further analysis of these samples demonstrated that there was a statistically significant association between the presence of the *T. phagedenis* DD treponeme phylogroup in the rectum and the housing season (Oct to March) (Evans et al., 2012b).

Furthermore with regards to the GI tract, Evans et al., (2012b) found the rumen at the reticular pillar and the dorsal sac to be positive by PCR for the *T. phagedenis* DD treponeme phylogroup in a cow which was also positive in the gingiva. Other studies have also detected DD treponeme DNA in the rumen fluid (Nascimento et al., 2015; Zinicola et al., 2015b), with one study reporting the treponemes detected to be ubiquitous to those reported in the DD lesions also investigated (Zinicola et al., 2015b). The presence of DD treponemes in the GI tract ties in with nutrition and hygiene as risk factors for DD, as well

as the buying in of cattle which may unknowingly be infected with DD treponemes in the GI tract.

Table 1.4: Investigation of potential infection reservoir sites for DD treponemes in relation to dairy cattle by molecular and isolation methods

Location of sites investigated for infection reservoirs	Sites in which DD treponemes have been <u>detected</u>	Sites in which the detection of DD treponemes have <u>failed</u>
Dairy cattle tissues	• Gingiva adjacent to upper molar[1] • Rumen at reticular pillar [1] • Rumen dorsal sac [1] • RAJ [1] • Rectal wall [1] • Rumen fluid [2, 3] • Skin above the hind leg hock [1] • Hind foot (DD-unaffected) skin between the bulbs of the heel [1, 6] • Fore foot skin between the bulbs of the heel[1]	• Rumen content [1] • Internal mucosa of nostril [1] • Lips [1] • Buccal mucosa [1] • Gingiva adjacent to lower molar [1] • Tongue [1] • Oesophagus [1] • Rumen ventral sac [1] • Omasum [1] • Abomasum (fundic and pyloric) [1] • Duodenum [1] • Jejunum [1] • Illeum [1] • Caecum [1] • Colon [1] • Peri-anal skin [1] • Perineal skin [1] • Skin below tail/ above anus[1] • Urethra [1] • Bladder wall [1] • Spleen [1] • Kidney [1] • Pancreas [1] • Liver [1] • Lung [1] • Mesenteric lymph node [1] • Hind leg inner thigh skin [1] • Hind fetlock skin [1] • Skin below hock [1]
Environment	• Faeces [3, 4] • Slurry [4, 5] • Foot trimming knife blades [7]	• Urine [1] • Diptera (*Musca autumnalis, Pyschodidae, Stomoxys calicitrans*) [1] • Faeces [1, 8] • Slurry [1]

[1] Evans et al., 2012 [2] Nascimento et al., 2015, [3] Zinicola et al., 2015, [4] Klitgaard et al., 2014, [5] Klitgaard et al., 2017 and [6] Evans et al., 2009c, [7] Sullivan et al., 2014 and [8] Nordhoff, 2006.

In a small number of animals DD treponemes have also been detected by PCR in macroscopically healthy pedal skin tissue between the bulbs of the heel of the plantar/ palmer aspect of the hind/ forefeet and in one case in the skin above the hock of one of the hind legs (Evans et al., 2009c, 2012b). Thus DD treponemes may also be able to colonise healthy skin and further investigation would be required to delineate these findings.

1.10.2 Infection reservoirs within the dairy farm environment

The discovery of DD treponemes in the GI tract and hygiene as a risk for DD strongly suggests that DD treponemes may be present in bovine faeces and slurry. Initial investigation into faeces and slurry as an infection reservoir for DD failed to detect DD treponemes in these environmental samples (Nordhoff, 2006; Evans et al., 2012b). However, recent metagenomic studies have detected DD treponemes in both faeces and slurry (Table 1.4) (Klitgaard et al., 2014, 2017; Zinicola et al., 2015b).

Fomites may also play a role in DD treponeme infection after the discovery of DD treponemes on the blades of foot trimming knives and gloves following foot trimming of cattle and sheep and the handling of CODD-affected feet respectively (Sullivan et al., 2014a; Angell et al., 2017). With the foot trimming knives, 62%, 57% and 54% of blades were PCR positive for the *T. medium*, *T. phagedenis* and *T. pedis* DD treponeme phylogroups respectively (Sullivan et al., 2014a). In addition, a treponeme belonging to the *T. phagedenis* phylogroup was isolated by cultivation from one of the blades which suggests treponemes on foot trimming knife blades are viable and thus transmissible (Sullivan et al., 2014a). Indeed, 100% of gloves used to handle clinical cases of CODD were PCR positive for one or more of the DD treponeme phylogroups and 91% of these samples were also positive by culture (Angell et al., 2017).

This data provides evidence for risk factors involving foot trimming cited by Wells et al., (1999) as it is likely that DD treponemes are passed from cow to cow via the foot trimming knife and contaminated gloves on and between farms. In addition it may explain why a risk factor study found longer intervals between foot trimming was associated with a reduced risk of DD, as these cows will not be exposed to the treponemes on the knife or gloves as frequently as on other operations (Holzhauer et al., 2006). The presence of DD treponemes on these fomites is highly suggestive that they could also be present on other types of fomites.

Disinfection of the hoof knife blades with an Department for Environment, Food and Rural Affairs (DEFRA) approved 2.5% (w/v) available iodine disinfectant (product name not specified) failed to completely eliminate detection of the DD treponemes by PCR (Sullivan et al., 2014a). Whereas disinfection of the gloves with either a 1:90 dilution of FAM (iodine based disinfectant) or 70% (v/v) ethanol eliminated detection of DD treponemes by culture and PCR, although various other methods of disinfecting the gloves of DD treponemes either made no effect (e.g. washing with warm water) or only reduced detection (e.g. water and hand soap) by culture and/ or PCR (Angell et al., 2017). Thus this highlights the importance of using a disinfection method tailored to need i.e. the treponemes associated with DD.

1.10.3 Other species and infection types in DD spread

Recently, MLST was used to delineate whether the same sequence types (STs) of DD treponemes were present in lesions from different species (Clegg et al., 2016b). This study showed that it was indeed possible for the same STs, and therefore what could be considered the same strains, from each of the three DD treponeme phylogroups to be found within lesions from different species (Clegg et al., 2016b). Therefore, this is highly suggestive that cross-species transmission events are possible, which may explain the finding that the presence of cattle with DD on the same farm as sheep is a risk factor for CODD (Angell et al., 2014). This knowledge and the fact that DD treponemes can be found viable on fomites is especially worrying for mixed farms and professionals in the livestock industry who may travel between farms dealing with different livestock species. It is imperative the role of fomites is further understood for tighter biosecurity measures.

Additionally the presence of DD treponemes in other 'non-healing' foot disorders and on foot trimming knives following trimming of DD-unaffected feet means that vigilance is required even if DD lesions are not necessarily present when foot trimming (Evans et al., 2011a). Other infections in dairy cattle such as pressure sores (Clegg et al., 2016d), hock lesions (Clegg et al., 2016a), ulcerative mammary dermatitis (Evans et al., 2010) and the severely debilitating ischaemic teat necrosis (Clegg et al., 2016c) that may also contain DD treponemes must be considered when controlling DD as it is highly likely they will also act as reservoirs enabling maintenance of DD of farm.

1.11 Aims of the project

Currently, treatment and control strategies are not capable of eliminating DD on farm and thus farms remain endemically infected. An alternative method of control would be to prevent initial infection. In order to understand and prevent transmission it is important to identify the infection reservoirs of DD treponemes. The project aims are therefore to:

1. Improve techniques for the molecular detection of DD treponemes in bovine faeces:

    - Optimise the *T. medium, T. phagedenis* and *T. pedis* phylogroup specific nested PCR assays currently used for the detection of DD treponemes for use with bovine faecal samples

    - Optimise a DNA extraction technique from bovine faeces that will enable the downstream detection of DD treponemes by PCR

2. Identify potential infection reservoirs of DD treponemes in the dairy cow and dairy farm environment:

    - Build upon the previous work by Evans et al., (2012b) by surveying a larger number of dairy cattle for the detection of DD treponemes in the gingiva, RAJ and healthy pedal tissue and to determine if there are any temporal associations of DD treponemes with these tissues

    - To survey the dairy farm environment for the presence of DD treponemes in faeces, feed, water, gloves, foot trimming tools and footprints

3. Further characterise the carriage of DD treponemes in dairy cattle tissues other than DD lesions:

    - Determine if carriage in tissues can occur without disease and determine the localisation of treponemes in these various tissues using histopathology and immunohistochemistry

    - Investigate whether the same DD treponeme phylogroup STs found previously in DD lesions from various species (Clegg et al., 2016b) are able to colonise other tissue types from different anatomical sites

4. To determine the growth and survival of DD treponemes under different conditions relating to the host and dairy farm environment:

    - To conduct survival studies to investigate DD treponeme survival and growth at different temperatures, different pH values, in bovine faeces and different types of bedding typically used in dairy systems

Chapter 2: General materials and methods

This chapter details the specific materials and methods used throughout this thesis.

2.1 Media and supplements

Liquid and solid media, as well as media supplements, used to culture treponemes routinely and for isolation throughout this thesis are listed in Table 2.1 along with their preparation.

Table 2.1: Media and supplements used for treponeme culture and isolation

Media/ Antibiotic	Preparation
Culture medium for *T. medium* phylogroup (Evans et al., 2008)	For routine culture: oral treponeme enrichment broth (OTEB; Anaerobe Systems, Morgan Hill, California, USA) was supplemented with 10% (v/v) rabbit serum (RS; GE Healthcare Life Sciences, Buckinghamshire, UK). For isolation from samples: OTEB 10% (v/v) RS was further supplemented with 5 or 25 µg/ml rifampicin and 5 µg/ml enrofloxacin (plus equivalent volume of enrofloxacin balance solution).
Culture medium for *T. phagedenis* phylogroup and *T. pedis* (Evans et al., 2008)	For routine culture: oral treponeme enrichment broth (OTEB; Anaerobe Systems, Morgan Hill, California, USA) was supplemented with 10% (v/v) foetal calf serum (FCS; Gibco, Paisley, UK). For isolation from samples: OTEB 10% FCS was further supplemented with 5 or 25 µg/ml rifampicin and 5 µg/ml enrofloxacin (plus equivalent volume of enrofloxacin balance solution).
Defibrinated sheep blood	Defibrinated sheep blood (20 ml) was obtained from Oxoid Ltd, Basingstoke, UK and stored at 4 °C.
Enrofloxacin (10 mg/ml)	50 mg of enrofloxacin (Sigma-Aldrich, Dorset, UK) was dissolved into 1M potassium hydroxide (KOH) (Sigma-Aldrich, Dorset, UK), filter sterilised using a 0.22 µm syringe filter (Sartorius, Surrey, UK), protected from light and stored in 500 µl aliquots at 4 °C.
Enrofloxacin balance solution	1 M hydrochloric acid (HCL) (Sigma-Aldrich, Dorset, UK) was filter sterilised 0.22 µm syringe filter (Sartorius, Surrey, UK) and stored in 100 µl aliquots at 4 °C. An equivalent volume of 1M HCL was added to culture medium when enrofloxacin stock solution was used so as to rebalance the pH of the culture medium.
Foetal calf serum (FCS)	FCS (Gibco, Paisley, UK) was heat inactivated in a 56 °C water bath for 30 minutes and 20 ml aliquots were stored at -20 °C.
Rabbit serum (RS)	RS (GE Healthcare Life Sciences, Buckinghamshire, UK) was heat inactivated in a 56 °C water bath for 30 minutes and 20 ml aliquots were stored at -20 °C.

Rifampicin (5 mg/ml)	50 mg of rifampicin (Sigma-Aldrich, Dorset, UK) was dissolved into 10 ml of methanol (analytical grade), filter sterilised using a 0.22 µm syringe filter (Sartorius , Surrey, UK) and stored in 1 ml aliquots at -20 °C.
Solid media for *T. medium* phylogroup (Evans et al., 2008)	For routine culture: fastidious anaerobe agar (FAA; Lab M, Bury, UK), was prepared according to manufacturer's instructions, allowed to cool to 50°C after autoclaving and subsequently supplemented with 5% (v/v) defibrinated sheep blood (Oxoid Ltd, Basingstoke, UK), and 10% (v/v) rabbit serum (RS; GE Healthcare Life Sciences, Buckinghamshire, UK). For isolation from samples: FAA was further supplemented with with 5 or 20 µg/ml rifampicin and 5 µg/ml enrofloxacin (plus equivalent volume of enrofloxacin balance solution).
Solid media for *T. phagedenis* phylogroup and *T. pedis* (Evans et al., 2008)	For routine culture: fastidious anaerobe agar (FAA; Lab M, Bury, UK), was prepared according to manufacturer's instructions, allowed to cool to 50°C after autoclaving and subsequently supplemented with 5% (v/v) defibrinated sheep blood (Oxoid Ltd, Basingstoke, UK), and 10% (v/v) foetal calf serum (FCS; Gibco, Paisley, UK). For isolation from samples: FAA was further supplemented with with 5 or 20 µg/ml rifampicin and 5 µg/ml enrofloxacin (plus equivalent volume of enrofloxacin balance solution).
Transport medium	Oral treponeme enrichment broth (OTEB) (Anaerobe Systems, Morgan Hill, California, USA) supplemented with 5 µg/ml rifampicin and 5 µg/ ml enrofloxacin, aliquoted into 2 ml screw cap tubes and stored at 4 °C.

2.2 Buffers and reagents

Buffers and reagents used throughout this thesis are listed in Table 2.2 along with their preparation.

Table 2.2: Buffers and reagents used for various studies in this thesis

Buffer/ reagent	Preparation
Agarose (1% w/v)	1 g of agarose (Bio-Rad, Hemel Hempstead, UK) was dissolved in 100 ml of 1X TAE buffer by heating and allowed to set.
Ammonium persulphate (10% w/v)	1 g of ammonium persulphate (Sigma-Aldrich, Dorset, UK) was dissolved in 10ml of ddH_2O to make a 10% (w/v) solution. Stored at 4°C and replaced every 2-3 weeks.
Chelex-100 resin (5% w/v)	5 g of Chelex-100 resin (Bio-Rad, Hemel Hempstead, UK) was added to 10 ml of ddH_2O.
dNTPs (20mM)	A stock solution of dATP, dCTP, dGTP and dTTP at 5 mM each was obtained from Thermo Scientific, Hemel Hempstead, UK and 100 µl aliquots were stored at -20°C.
Ethyleneglycol tetraacetic acid buffer; EGTA (100mM)	0.380 g of EGTA (Sigma-Aldrich, Dorset, UK) was dissolved in 1M NaOH and filter sterilised.
Ethidium bromide (EtBr)	10 mg/ ml of EtBr in ethanol was obtained (Bio-Rad, Hemel Hempstead, UK)
Glycerol	10 ml aliquots of glycerol (BDH, Dorset, UK) was sterilised by

	autoclaving.
Magnesium chloride; $MgCl_2$ (5mM)	A 100 mM stock solution of $MgCl_2$ (Sigma-Aldrich, Dorest, UK) was prepared by dissolving 0.203 g of $MgCl_2$ in 10 ml 1X PBS. A working concentration of 5 mM $MgCl_2$ was then prepared by adding 5 ml of 100 mM $MgCl_2$ stock solution to 95 ml of 1X PBS. Filter sterilised.
Marvel (5% w/v)	5g of Marvel (Chivers, Dublin, ROI) dissolved in 100 ml PBST
Phosphate buffered saline (PBS) (1X)	5 phosphate buffered saline tablets (Sigma-Aldrich, Dorset, UK) were dissolved in 1 L of ddH_2O to make 1X PBS, pH 7.4. If necessary sterilised by autoclaving.
Phosphate buffered saline with Tween® 20; PBST (0.05% v/v)	500 µl of Tween® 20 (BDH, Dorset, UK) was added to 1L of 1X PBS.
Resolving gel (12% v/v)	10 ml of 12% (v/v) resolving gel was prepared as follows: 3.3 ml ddH_2O, 4 ml of 30% (w/v) acrylamide, 2.5 ml of 1.5M Tris-HCl (pH8.8), 0.1 ml of 10% (w/v) ammonium persulphate, 0.1 ml of 10% (w/v) SDS and finally just before casting the gel 4 µl of TEMED.
Sodium dodecyl sulphate; SDS (10% w/v)	10g of SDS was dissolved in 80 ml ddH_2O. The volume was adjusted to 100 ml with ddH_2O.
Sodium chloride; NaOH (1M)	4 g of NaoH (Sigma-Aldrich, Dorset, UK) dissolved in 100 ml ddH_2O.
Stacking gel (5% v/v)	4 ml of 5% (v/v) stacking gel was prepared as follows: 2.7ml ddH_2O 0.67 ml of 30% (w/v) acrylamide, 0.5 ml of 1M Tris-HCl (pH 6.8), 40µl of 10% (w/v) SDS, 40 µl 10% (w/v) ammonium persulphate and finally just before casting gel 4 µl of TEMED.
TAE buffer (1X)	4 L of 1X TAE buffer was prepared by adding 100 ml of 40 X TAE buffer (molecular grade) (Sigma-Aldrich, Dorset, UK) to 3900 ml of dd H_2O.
Tetramethylethylenediamine (TEMED)	Catalyst for polyacrylamide gel polymerisation (Thermo Scientific, Hemel Hempstead, UK).
Transfer buffer	Transfer buffer was prepared by adding 3.03 g trizma base (Sigma-Aldrich, Dorset, UK), 14.4 g glycine (Sigma-Aldrich, Doreset, UK), and 200 ml methanol (analytical grade) (Thermo Scientific, Hemel Hempstead, UK) together and adjusting the volume of the solution to 1 L with ddH_2O.
Tris-glycine running buffer (5X)	To prepare a 5X stock solution of Tris-glycine running buffer 15.1 g trizma base (Sigma-Aldrich, Dorset, UK), 94 g glycine (electrophoresis grade) (Sigma-Aldrich, Dorset, UK) and 50 ml 10% (w/v) SDS were dissolved in 1L of ddH_2O. A 1X working solution prepared from the 5X stock solution was used for electrophoresis.
Tris-HCl (1M, pH 6.8)	A 1M solution of Tris-HCl was prepared by dissolving 121.1 g of Trizma base (Sigma-Aldrich, Dorset, UK) in 800 ml ddH_2O. The pH was adjusted to pH 6.8 with the addition of concentrated HCl. The volume was adjusted to 1 L. The solution was aliquoted and sterilised by autoclaving.
Tris-HCl (1.5M, pH 8.8)	A 1.5M solution of Tris-HCl was prepared by dissolving 181.7 g of Trizma base (Sigma-Aldrich, Dorset, UK) in 800 ml ddH_2O. The pH was adjusted to pH 8.8 with the addition of concentrated HCl. The volume was adjusted to 1 L. The solution was aliquoted and sterilised by autoclaving.

2.3 Farm information

The farm details from which environment samples (faeces, mucin casts, feed, water, foot trimming equipment, footprints and bedding) were collected are listed in Table 2.1.

Table 2.3: Farm information[a]

Farm	County	Herd size[b]	Daily milking frequency	Summer grazing access?	Parlour floor surface	Crush floor surface	DD footbath prevention
A	Cheshire	220	3	No	NT	Metal	Either formalin once weekly or copper sulphate twice weekly
B	Glouc	300	2	Yes	Rubber	Rubber*	2x Daily at parlour exit
C	Glouc	450	3	No	Concrete	Rubber*	2x Daily at parlour entrance
D	Glouc	220	2	Yes	Rubber	NT	2x Daily at parlour exit
E	Worcs	450	3	No	NT	Rubber*	2x Daily at parlour exit
F	Glouc	-	-	-	NT	NT	-
G	Cheshire	-	-	-	NT	NT	-

[a] *Abbreviations: Glouc, Gloucestershire; Worcs, Worcestershire; NT, not tested. (-) denotes not known.*

[b] *Herd size is an approximation.*

** Foot trimmers crush.*

2.4 Bovine faecal samples

Bovine faecal samples were collected from dairy farms where DD was endemic. Fresh faecal samples were collected immediately after defecation, either by collecting the faeces as it fell during defecation or by taking a sample from faeces once it had fallen to the ground, taking care to avoid cross contamination with the ground. Initially faecal samples were from dairy cattle of unknown DD status. However, subsequent collection occurred from dairy cattle whose feet were lifted and checked for DD lesions by an attending veterinary surgeon at the time of collection or by collecting from cows that had recently been identified as having DD on the farm records. Approximately 30- 50 g of faeces was collected per sample. For a small number of samples, 1 g of faeces was transferred into transport medium (Table 2.1) for subsequent culturing. The remainder of the sample was

transported on ice and stored at -20˚C. Further details of sample numbers can be found in Chapter 4 Section 4.2.2.

2.5 Treponeme cultivation

2.5.1 Cultivation of isolated digital dermatitis treponemes in liquid media

The DD-associated treponeme strains including *T. medium* phylogroup strains T19 and T56, *T. phagedenis* phylogroup strains T320A and T354B and *T. pedis* strains T3552B and T354A, previously isolated by this lab and stored in 10% (v/v) glycerol at -80°C were thawed and transferred into an anaerobic cabinet (85% N_2, 10% H_2 and 5% CO_2, 36 °C) for inoculation. Using sterile glass pasteur pipettes (VWR International Ltd, Leicestershire, UK) 10 drops (~300µl) of the *T. phagedenis* phylogroup strains or *T. pedis* strains were inoculated into OTEB 10% (v/v) FCS (Table 2.1). For *T. medium* phylogroup strains approximately 15 drops (~450µl) of thawed culture were inoculated into OTEB 10% (v/v) RS (Table 2.1). Cultures were checked for treponeme growth on days 4 and 7. Growth was observed via phase contrast microscopy where spiral morphology could be observed with rotational and translational motility as well as flexing and jerking movements (Evans et al., 2009b). Appearance (i.e. size, morphology etc) of the treponemes in culture (examples in Figure 2.1) could also be used to help confirm phylogroup present (Evans et al., 2008).

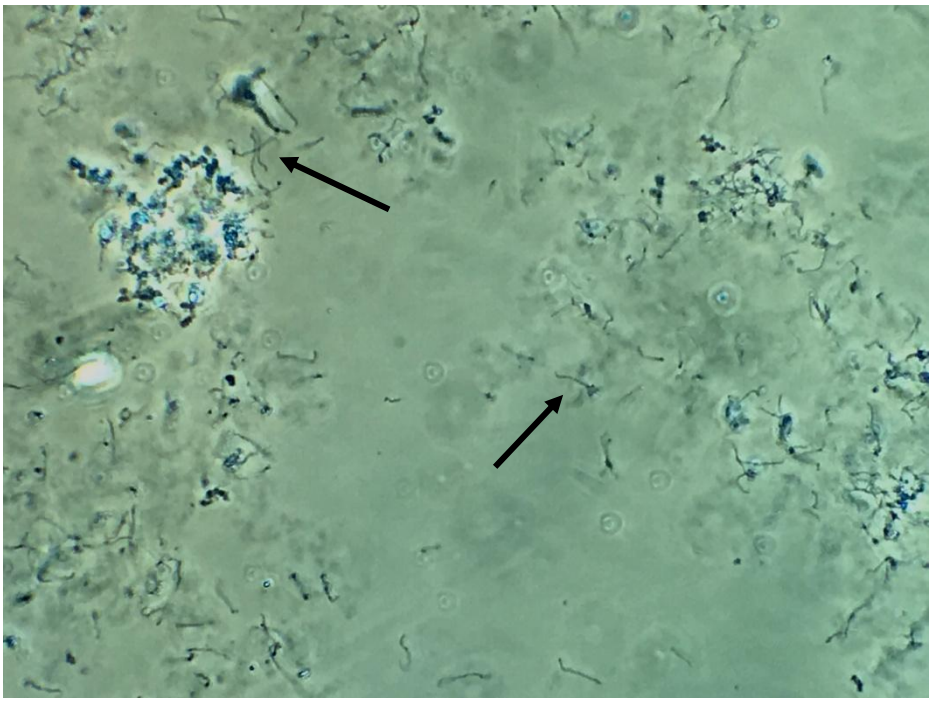
After sufficient growth, which could take between 7 and 10 days from glycerol culture stocks, treponemes were subcultured into fresh liquid media. For treponemes belonging to either the *T. phagedenis* phylogroup or *T. pedis* phylogroup, 3 drops (~90µl) of culture were inoculated into OTEB 10% (v/v) FCS. For treponemes belonging to the *T. medium* phylogroup, nine drops (~270µl) of culture were inoculated into OTEB 10% (v/v) RS. Cultures were then maintained by subculturing every 4 days for *T. pedis* strains and every 7 days for treponemes belonging to *T. medium* and *T. phagedenis* phylogroups.

2.5.2 Cultivation of digital dermatitis treponemes on solid media

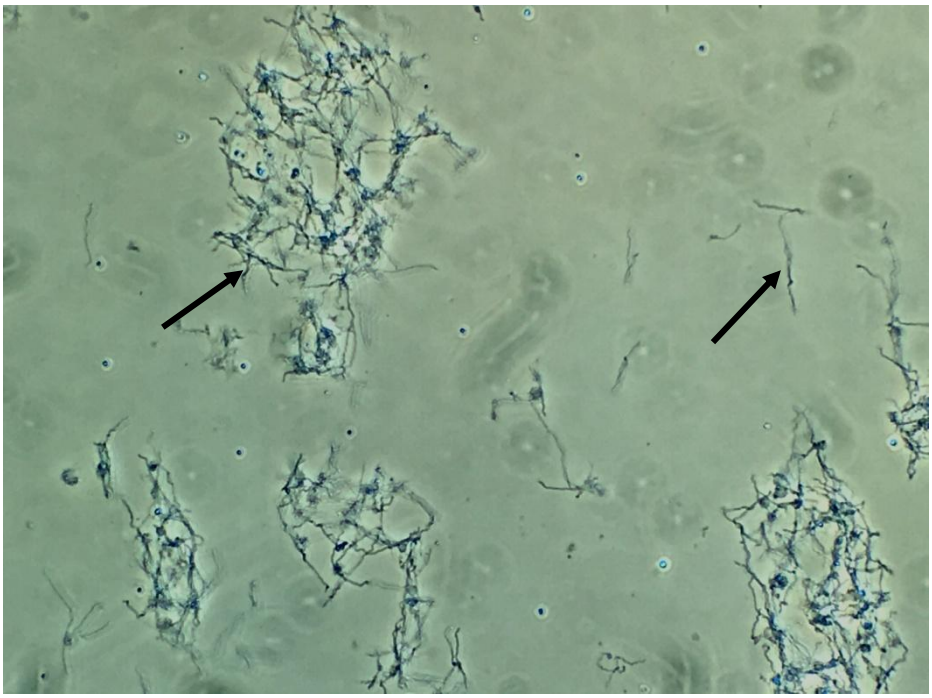
After 2-7 days of good growth in liquid media, treponemes could be sub-cultured onto FAA blood 10% (v/v) FCS plates or FAA blood 10% (v/v) RS plates depending upon DD treponeme phylogroup present. Plates were streaked with 1-2 drops (~30-60µl) of treponeme liquid culture and the plate was approximately three quarters sealed with tape to prevent plates drying out too quickly. Both inoculations and incubation were under anaerobic conditions (85% N_2, 10% H_2 and 5% CO_2, 36 °C) and after 1-2 weeks single colonies can be visualised on the plate as described by Evans *et al.*, (2008, 2009b). Single

colonies could then be inoculated back into OTEB supplemented with either 10% (v/v) FCS or RS depending on the phylogroup. After sufficient incubation, cultures could be checked for growth and purity by phase contrast microscopy and sub-cultured as necessary.

a)



b)



c)

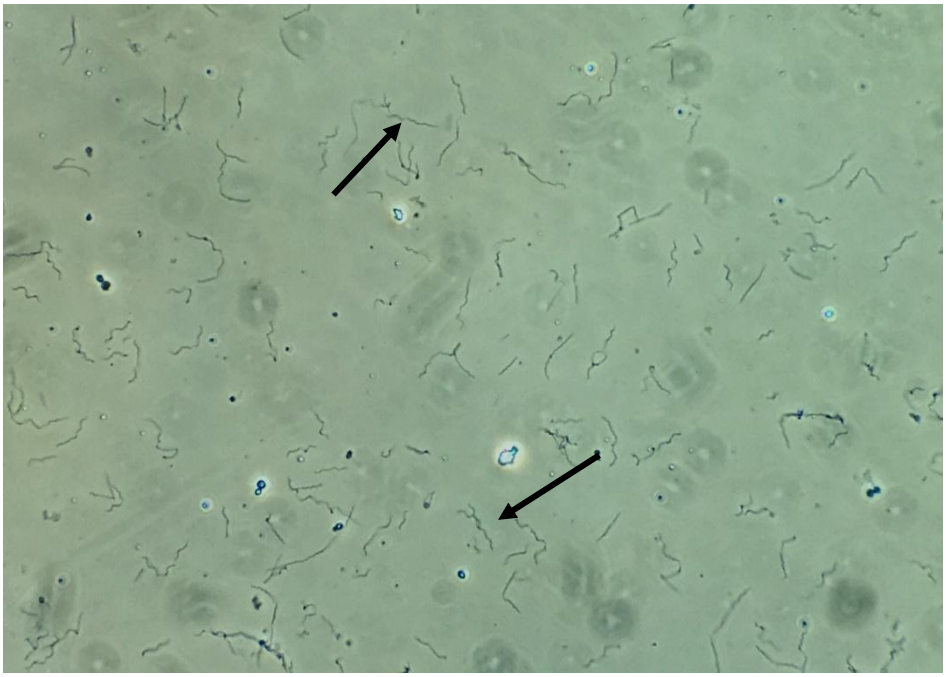


Figure 2.1: Examples of DD treponeme phylogroup cultures via phase contrast microscopy

Figure 2.1 Phase contrast microscopy images of a) an example of a T. medium phylogroup strain T56 culture, b) an example of a T. phagedenis phylogroup strain T354B culture and c) an example of T. pedis culture. Arrows point to examples of individual characteristic spirochaete morphology within each culture. Examples of characteristic clumping of multiple treponemes can be observed in a) and b). 40x magnification. No scale available due to method of photography. Figure source: authors photograph taken through microscope lens.

2.5.3 Cultivation of isolated commensal GI tract treponemes in liquid media

Commensal GI tract treponemes previously isolated by this lab and stored in 10% (v/v) glycerol at -80°C were thawed and transferred into an anaerobic cabinet (85% N_2, 10% H_2 and 5% CO_2, 36 °C) for inoculation. Using sterile glass pasteur pipettes (VWR International Ltd, Leicestershire, UK) approximately 15 drops (450 µl) were inoculated into OTEB. Cultures were checked every 1-2 days for treponeme growth as per the growth requirements of commensal GI treponemes (Evans et al., 2011b; Newbrook et al., 2017; Staton et al., 2017). Growth was observed via phase contrast microscopy as described in Section 2.5.1. After sufficient growth, which could take as little as 1 day from glycerol culture stocks, treponemes were sub-cultured into fresh liquid media. For subculture, 10-15 drops (300-450 µl) of each culture were inoculated into a new tube of OTEB. Every 1-3 days subcultures were checked and sub-cultured for culture maintenance.

2.5.4 Cultivation of commensal GI tract treponemes on solid media

Following growth in OTEB, commensal GI treponemes could be sub-cultured onto FAA plates (Table 2.1), as described in Section 2.5.2. After approximately 5 days growth single colonies can be visualised as described by Evans et al., (2011). Single colonies could then be inoculated into OTEB. Following sufficient growth, cultures could be checked for growth and purity by phase contrast microscopy and sub-cultured as necessary.

2.5.5 Treponeme culture storage

Upon subculturing a 2 ml aliquot of treponeme culture is stored at -80°C in autoclaved 10% (v/v) glycerol. Furthermore the culture is occasionally aliquoted into a 1.5 ml tube and stored at -20°C for subsequent DNA extraction and downstream applications.

2.6 DNA extraction

2.6.1 Treponeme culture DNA extraction

Treponeme cultures which had been stored at -20°C were thawed on ice and DNA extracted using a Chelex resin method (Chua et al., 2005). Briefly, 1.5 ml of culture was centrifuged at 15 000 rpm for 3 minutes at room temperature using a bench top centrifuge (Labnet Prism™ microcentrifuge, Labnet International, Cary, NC, USA). Approximately half of the supernatant was discarded and the cell pellet resuspended. The resuspended pellet was boiled for 10 minutes with 250 µl of 5% (w/v) Chelex-100 resin (Bio-Rad, Watford, UK). After cooling the extracted DNA was centrifuged for 10 minutes at 13 000 rpm at room temperature. The supernatant which contains the purified DNA was aliquoted and stored at -20˚C for downstream applications.

2.7 Polymerase chain reaction (PCR) assays

2.7.1 Universal bacterial 16S rRNA gene PCR assay

The majority of the 16S rRNA gene was amplified using a universal bacterial primer pair (Table 2.4) (Rurangirwa et al., 1999). Template DNA (1 µl) was added to 24 µl PCR reaction mix per sample. The PCR reaction mixes included 0.125 µl of *Taq* polymerase (Qiagen, Manchester, UK) combined with relevant components according to the manufacturer's instructions, 1 µl of 20mM dNTPs stock solution (Thermo Scientific, Hemel Hempstead, UK), 2.5 µl of 10X buffer (Qiagen, Manchester, UK), 5 µl of Q buffer (Qiagen, Manchester, UK), 1 µl each of forward and reverse primers (0.1 mM stock solutions) and 13.4 µl of molecular

grade double distilled (dd) H_20. The PCR thermocycling used a Biometra TRIO thermocycler (Thistle Scientific, Glasgow, UK) with conditions of 95˚C for 5 minutes followed by 94˚C for 1 minute, 55˚C for 3 minutes, 72˚C for 3 minutes for 40 cycles and a final extension stage of 72˚C for 7 minutes.

2.7.2 Digital dermatitis treponeme phylogroup specific 16S rRNA gene nested PCR assay

The DD treponeme phylogroup specific 16S rRNA gene nested PCR assays involved two steps: an initial universal bacterial 16S rRNA gene PCR step and a second DD treponeme phylogroup specific nested step (Evans et al., 2009c). The initial universal bacterial 16S rRNA gene step amplified the majority of the 16S rRNA gene with the same PCR reaction mix and PCR cycling conditions as described above (Section 2.7.1) except that 25 cycles were employed rather than 40 cycles.

The second nested step utilises three sets of DD treponeme phylogroup specific primers (Table 2.4) which amplify 300-500bp sequences within the universal bacterial 16S rRNA gene PCR product. The three targeted DD treponeme phylogroups are *T. medium*, *T. phagedenis* and *T. pedis.* One µl of the PCR product from the initial 16S rRNA gene step was added as the template to a 24 µl PCR reaction mix. The components of the PCR reaction mix for the second nested step is the same as for the bacterial universal 16S rRNA step except for using the aforementioned phylogroup specific primers. The cycling conditions were an initial step of 95˚C for 5 minutes followed by 40 cycles of 95˚C for 1 minute, 68˚C for 1 minute for *T. medium* phylogroup specific primers / 64˚C for 1 minute *T. phagedenis* phylogroup specific primers / 67°C for 1 minute for *T. pedis* phylogroup specific primers, 72˚C for 2 minutes and lastly a final extension of 72˚C for 10 minutes.

Extracted genomic DNA from each DD treponeme phylogroup (*T. medium* strain T19 or T56, *T. phagedenis* strain T320A or T354B and *T. pedis* strain T3552B or T354A) were included in each assay to act as positive or negative controls depending upon which phylogroup specific primers were used. Molecular grade water was used as a negative control in each assay. Aerosol-resistant tips, separate processing areas and regular changing of gloves were employed to minimise the possibility of cross-contamination, which is a high risk with nested PCR assays due to working with PCR product as a template. Additionally, assays were carried out in triplicate on different occasions to ensure result repeatability and reliability as previously reported for other DD treponeme detection studies and other pathogen detection assays (Nakamura et al., 1999; Kuoppa et al., 2002; Rougemont et al.,

2004; Langrell, 2005; Clegg et al., 2015, 2016a; c; d; e; Crosby-Durrani et al., 2016), with the median result reported.

2.7.3 *Treponema* genus specific 16S rRNA gene PCR assay

A *Treponema* genus specific primer set (Table 2.4) was utilised that amplified a small region of the 16S rRNA gene which would allow detection of all commensal and pathogenic *Treponema* species (Moore et al., 2005). The assay was carried out using 1 µl of DNA template in a 24 µl PCR reaction mix. The PCR reaction mix included 0.25 µl of *Taq* polymerase (Qiagen, Manchester, UK), 1 µl of dNTPs (20 mM stock solution; Thermo Scientific, Hemel Hempstead, UK), 1.5 µl of $MgCl_2$ (Qiagen, Manchester, UK), 2.5 µl of 10X buffer (Qiagen, Manchester, UK), 5 µl of Q buffer (Qiagen, Manchester, UK), 0.4 µl of forward and reverse primers (0.1 mM stock solutions) and 13 µl of ddH_2O. Thermocycling conditions were 35 cycles of 95°C for 15 seconds, 53°C for 30 seconds, 72°C for 30 seconds followed by final elongation at 72°C for 5 minutes as originally described (Moore et al., 2005).

Table 2.4: PCR assays primers

Primer specificity	Primer sequence (5'-3') (forward and reverse)	16S rRNA gene position[a]	Band size (bp)	Reference
Universal 16S rRNA gene	AGAGTTTGATCCTGG	7-26	1526	(Rurangirwa et al., 1999)
	TACCTTGTTACGACTT	1491-1506		
T. medium phylogroup	GAATGCTCATCTGATGACGGTAATCGACG	472-500	475	(Evans *et al.*, 2009c)
	CCGGCCTTATCTAAGACCTTCTACTAG	1001-1029		
T. phagedenis phylogroup	GAAATACTCAAGCTTAACTTGAGAATTGC	612-640	400	(Evans et al., 2009c)
	CTACGCTACCATATCTCTATAATATTGC	1006-1029		
T. pedis phylogroup	GGAGATGAGGGAATGCGTCTTCGATG	459-484	475	(Evans *et al.*, 2009c)
	CAAGAGTCGTATTGCTACGCTGATATATC	1017-1045		
Treponema genus	AARCATGCAAGTCGARCGGCAAG	49-71	335	(Moore et al., 2005)
	TCCATTGCGGAATATTCTTA	365-384		

[a] *16S rRNA gene positions relative to the Escherichia coli 16S rRNA gene sequence (Genbank accession: M25588)* (Ehresmann et al., 1975).

Extracted genomic DNA from DD treponeme phylogroups (*T. medium* strain T19 or T56, *T. phagedenis* strain T320A or T354B and *T. pedis* strain T3552B or T354A) were included as positive controls. Molecular grade water was used as a negative control in each assay. Assays were carried out in triplicate as described in Section 2.7.2.

2.7.4. Agarose gel electrophoresis

Results of PCR assays were visualised using agarose gel electrophoresis with a positive result indicated by the presence of a band at the correct size (Table 2.4) as determined by simultaneous comparison with DNA ladders. PCR product mixed in a 5:1 ratio with 6X Orange DNA loading Dye (Thermo Scientific, Hemel Hempstead, UK) (10 µl) was loaded into each well of a 1% (w/v) agarose gel (Bio-Rad, Watford, UK) stained with 0.5 mg/ml ethidium bromide to allow DNA visualisation and analysis (Bio-Rad, Watford, UK). At the opposite ends of each gel 4 µl of 1 Kb and 100 bp DNA ladders (Thermo Scientific, Hemel Hempstead, UK) were loaded for nucleic acid product size interpretation.

Loaded gels were immersed in 1X TAE electrophoresis buffer (Sigma-Aldrich, Dorset, UK) in a geneflow electrophoresis tank (GeneFlow Ltd, Staffordshire, UK) and run at 110 V using a Bio-Rad PowerPac 300 (Bio-Rad, Watford, UK). Ultraviolet light was used to visualise PCR product bands on the gel via a gel imaging system (Bio-Rad, Watford, UK).

2.8 16S rRNA gene sequencing

2.8.1 PCR purification

PCR products from the universal bacterial 16S rRNA gene PCR assay were purified using the QIAquick® PCR Purification kit (Qiagen, Manchester, UK) according to the manufacturer's instructions.

2.8.2 16S rRNA gene sequencing

Sequencing of purified PCR products was carried out commercially (Beckman Coulter, High Wycombe, UK/ Source Bioscience, Rochdale, UK/ Macrogen, Amsterdam, The Netherlands) using the universal 16S rRNA primer set described in Table 2.1. Subsequently ChromasPro 1.7.5 (Technelysium Pty Ltd, South Brisbane, Australia) was used to assemble the sequences obtained into double stranded consensus sequences. The completed sequence was then exported as a 'FASTA' file. The microorganism to which the sequence was most similar to could then be identified by performing a nucleotide Basic Local Alignment Search

Tool (BLAST) search (Altschul et al., 1990) of the 16S rRNA gene sequence 'FASTA' file against the National Centre for Biotechnology Information (NCBI) nucleotide database.

2.8.3 Phylogenetic tree analysis of 16S rRNA gene sequencing

Phylogenetic analysis of the 16S rRNA gene consensus sequences obtained were carried out in order to determine the relatedness of spirochaetes of interest to pathogenic and commensal treponemes. 16S rRNA gene consensus sequences were aligned using CLUSTALW implemented by MEGA 6 (Tamura et al., 2013). 16S rRNA gene sequences of relevant spirochaetes were obtained from GenBank (Benson et al., 2013) and included in the analysis. The model test was performed by the Topali programme to determine the most suitable evolution model (Milne et al., 2009). Bootstrapping was preformed 10 000 times on the nucleotide maximum-likelihood tree chosen.

2.9 Ethical approval

Ethical approval for this project was obtained from the University of Liverpool ethics committee (application number VREC157).

Chapter 3: Development of optimised techniques for the detection of DD treponeme DNA from bovine faeces

3.1 Introduction

The shedding of pathogens in faeces is an important transmission route for a multitude of diseases in both humans and animals, including livestock. Cattle are known to shed a number of pathogens in their faeces which may either subsequently cause disease within the cattle herd or act as a natural source of infection for other host species. Examples of pathogens shed through bovine faeces include *Escherichia coli* O157: H7 a cause of severe gastroenteritis in humans (Chapman et al., 1989; Montenegro et al., 1990) and *Mycobacterium avium* subsp. *paratuberculosis* the cause of Johne's disease in cattle and sheep (Crossley et al., 2005). Often, once the pathogen is shed in faeces subsequent transmission into naïve hosts occurs via a faecal-oral route where faecal matter contaminates food sources or parts of the body that comes into contact with the mouth.

Faecal shedding can occur when the pathogen is colonising the gastrointestinal tract (GI) either through carriage without causing changes to the tissue or at the site of disease. *E. coli* O157:H7 colonises the lymphoid follicle rich rectal-anal junction (RAJ) mucosa in cattle without clinical disease (Naylor et al., 2003). Anywhere between 1 and 50% of cattle in a herd can be colonised and shedding *E. coli* O157:H7 in their faeces at any one time (Lim et al., 2010). Studies attempting to isolate DD treponemes from various parts of the GI tract, including the rectum and/ or RAJ, failed and instead reported isolation of alternative treponeme phylotypes not reported to be involved with DD (Evans et al., 2011b, 2012b). Subsequently a study by Evans et al., (2012b) investigating infection reservoirs of DD in dairy cattle using PCR based methods, detected DD-associated treponemes in 14.8% of the RAJ tissue sampled from dairy cattle, thus suggesting potential for faecal shedding of DD-associated treponemes. However, using the same DD treponeme phylogroup specific PCR assays used on rectal tissue, DD treponemes could not be identified in faeces collected from several DD endemic farms (Evans et al., 2012b). In contrast, more recent studies have

identified a small percentage of DNA homologous to DD-associated treponemes in faeces and slurry using high throughput, deep sequencing techniques (Klitgaard et al., 2014, 2017; Zinicola et al., 2015b). For example, Klitgaard et al., (2014) identified sequences belonging to DD-associated treponemes in 0.22% and 0.75% of all treponemal sequences which had been specifically PCR amplified from bovine manure and slurry. It could therefore be hypothesised that cattle colonised at the RAJ with DD-associated treponemes may shed these treponemes in their faeces, albeit, the number shed may be low as suggested by this deep sequencing data.

Previous experimental methods for DD treponeme DNA extraction from faeces as described by Evans et al., (2012b) utilised the Qiagen QIAamp® DNA stool kit and eluted DNA was analysed by phylogroup specific nested PCR assays (Evans et al., 2009c) and a *Treponema* genus specific PCR assay (Moore et al., 2005). Whilst no DD treponemes were detected in the DNA extracted from 35 faecal samples, they were all positive for *Treponema* genus demonstrating that treponemes can be detected in faeces and there is the potential for DD treponeme DNA to be present in faeces, but current extraction and PCR based detection techniques may not be sensitive enough to detect it. The studies utilising deep sequencing to identify DD treponeme specific DNA in environmental samples (including faeces) extracted DNA from the faeces using either the QIAamp® DNA stool mini kit (Qiagen) with an initial homogenisation step (Klitgaard et al., 2014) or with the Powersoil DNA isolation kit (MO BIO laboratories)(Zinicola et al., 2015b) which differs to the Qiagen QIAamp® DNA stool mini kit in that it contains a bead beating step.

There are many barriers that must be overcome in order to successfully extract DNA and detect the desired microorganism in bovine faeces. Bovine faeces contains indigestible components of the cow's diet such as cellulose and lignin (Rapp, 2010). Decaying plant material found within faeces is thought to compete with DNA for adsorption onto silica membranes used in many DNA extraction techniques resulting in co-purification with DNA (Harry et al., 1999). This will not only affect yield but these co-purified materials can also act as inhibitors in PCR reactions either by binding to the DNA or interfering with the PCR reaction (Wilson, 1997). Many commercial DNA extraction kits now have steps that aim to remove PCR inhibitors found in faeces (e.g. the InhibitEx buffer in the QIAamp® DNA stool mini kit) and the effect of any PCR inhibitors found in eluted DNA samples can be reduced through dilution of eluted DNA used as template in PCR reactions or with use of PCR facilitators in PCR reaction mixes such as bovine serum albumin (BSA) (Kreader, 1996).

Furthermore the microbial content of bovine faeces may not be homogenous throughout the faeces therefore if a small sample of faeces is taken for DNA extraction, this sample may not be representative of the entire faecal microbiome. For example, *E. coli* O157 is found in higher numbers at the surface of faeces than in the inner core which is most likely due to the colonisation site of *E. coli* in the gut being at the RAJ (Naylor et al., 2003; Pearce et al., 2004; Robinson et al., 2005). Therefore it is important that the potential for a heterogenic distribution of DD-associated treponemes in faeces is considered when sampling.

PCR based methods successful for the detection of DD treponeme DNA in faeces using deep sequencing involve a single step PCR assay either amplifying the V4 hyper-variable region of the bacterial 16S rRNA gene (Zinicola et al., 2015b) or amplifying V3 and V4 regions of the 16S rRNA gene specific for six species of treponemes for which the primers were demonstrated to cross-react with many DD-associated treponemes (Klitgaard et al., 2014). The DD treponeme phylogroup specific nested PCR method, currently used by Evans et al. (2009c) and the author, involves an initial universal eubacterial step which amplifies the majority of the 16S rRNA gene (1526bp) followed by a second PCR step, employing primer pairs that bind within the initial amplified 16S rRNA gene region, and are specific for each of the three characterised DD treponeme phylogroups (Evans et al., 2009c). This method has successfully detected DD treponemes in ruminant DD lesions, oral cavity and rectal anal junction tissues and foot trimming equipment but not in faeces (Evans et al., 2009c, 2012b; Sullivan et al., 2014a). A limitation of this nested PCR method may be that the initial universal eubacterial amplification step may bias for selection of more prominent bacterial species in faeces than DD-associated treponemes. Given the DD-associated treponemes may be already present in low numbers, this bias could further reduce the likelihood of DD treponeme phylogroup specific nested primers coming into contact with their target DNA sequences during the second step PCR assays.

The data presented by Klitgaard et al., (2014) and Zinicola et al., (2015b) identifying DD-associated treponeme DNA sequences in faeces along with the evidence of DD-associated treponemes in the RAJ (Evans et al., 2012b) suggest that faeces could be an infection reservoir of DD and indicates a potential transmission route for DD that could be intercepted by targeted prevention strategies. Therefore the aims of this study was to a) optimise the DD treponeme phylogroup specific PCR assays for use with DNA extracted from bovine faeces and b) compare and optimise bacterial genomic DNA extraction

techniques from bovine faeces to maximise the likelihood of DD-associated treponeme detection. Such studies should underpin further faecal and environmental surveys to determine whether faeces is a viable source of DD transmission.

3.2 Materials and methods

3.2.1 Optimisation of digital dermatitis treponeme phylogroup specific polymerase chain reaction assays

The second, nested step of the three DD treponeme phylogroup specific nested PCR assays (see Chapter 2 Section 2.7.2) was optimised for use as a single step PCR assay to be used for the detection of DD treponemes in bovine faeces. The PCR reaction conditions and primers for each phylogroup remained the same as the second step of the DD phylogroup specific nested PCR assays (Chapter 2 Table 2.4) but the cycle number was optimised for use as a single step PCR assay. Cycle number optimisation was achieved by running each of the DD phylogroup specific PCR assays for 40, 45, 50 and 55 cycles. For each DD phylogroup specific PCR assay, three controls were used for each of the phylogroups which had been previously DNA extracted using a Chelex resin method (as described in Chapter 2 Section 2.6.1) and archived to act as positive or negative controls depending upon which DD treponeme phylogroup specific PCR assay was ran. The *T. medium* DD treponeme phylogroup control strains were T19, T56 and *T. medium*. The *T. phagedenis* DD treponeme phylogroup control strains were T320A, T345B and *T. phagedenis*. The *T. pedis* strain controls were T3552B^{T} and T354A with *T. denticola* acting as a negative control for this phylogroup as it is considered a different species (Evans et al., 2009b). To further validate the assay, one or more commensal GI treponemes, Ru1 or Oc1 (Evans et al., 2011b), were also used as negative controls as well as water for each DD treponeme phylogroup specific PCR assay. The PCR assays with the different cycle numbers were also compared between two machines (Biometra T3000 Thermocycler and Biometra TProfessional TRIO Thermocycler, Thistle Scientific Ltd, Glasgow, UK) where the RAMP rate varied (3°C/s and 6°C/s).

Upon selection of an optimum cycle number, the DD treponeme phylogroup specific PCR assays underwent sensitivity assays using archived 10-fold serial dilutions (10^{0}-10^{-6}) of known concentrations of T19 (*T. medium* phylogroup strain), T320A (*T. phagedenis* phylogroup strain) and T3552B (*T. pedis* strain) (8.75 x 10^{7} cells/ml, 1.14 x 10^{8} cells/ml and 2.69 x 10^{8} cells/ml respectively). The corresponding dilution series were tested using the

normal DD treponeme phylogroup specific nested PCR assay and the optimised single step DD treponeme phylogroup specific PCR assay for that phylogroup to see if sensitivities for the two assays were comparable. All PCR assays were carried out in triplicate.

3.2.2 *Treponema pedis* phylogroup polymerase chain reaction primer analysis and optimisation

The *T. pedis* phylogroup specific primers (Chapter 2 Table 2.4) were checked to ensure the primer sequences complemented the *T. pedis* sequences they were annealing to. The sequences of *T. pedis* phylogroup strains T3552B and strain T354A, *T. sp.* Ovine strain G179 and *T. denticola* ATCC 35405T (negative control) were aligned using CLUSTALW implemented by MEGA 6 (Tamura et al., 2013) and the relevant sequences were compared to the forward and reverse *T. pedis* specific primers. The annealing time for the *T. pedis* specific primers was increased from 30 seconds to 1 minute. Furthermore a gradient PCR assay was carried out to make sure the optimum annealing temperature was employed to ensure both *T. pedis* strains T3552B and strain T354A produced PCR products. Briefly, cycling conditions of the gradient PCR remained the same as for the single step *T. pedis* specific PCR assay (Chapter 2 Section 2.7.2 and Section 3.2.1) and an annealing temperature gradient was set up across 12 wells in the PCR machine that ranged +/- 5°C of the current annealing temperature of 68°C. *T. pedis* strains T3552B and strain T354A were compared to determine annealing temperature and *T. denticola,* as nearest relative, was included to ensure an annealing temperature was chosen that allowed the PCR assay to remain specific for *T. pedis* only.

3.2.3 Digital dermatitis treponeme detection faeces inhibition assays

Faeces from a DD negative cow that had previously been stored at -20°C, was serially diluted ten-fold (10^{0}-10^{-8}) and two-fold (0-1/256) into 1X phosphate buffered saline (PBS, pH 7.4). An equal volume of *T. phagedenis* phylogroup strain T320A culture containing approximately $1.14x10^{8}$ cells/ml (Evans et al., 2009a), as determined by absorbance measurement at 540nm using a spectrometer, was inoculated into each of the faecal dilutions. In the initial experiment, farm sampling conditions were replicated by incubating both the DD treponeme spiked ten-fold and two-fold faecal serial dilutions for 1 hour at ambient temperature followed by 3 hours on ice. In a subsequent experiment, only the DD treponeme spiked two-fold serial faecal dilutions were incubated at ambient temperature for 2 hours, 4 hours, 6 hours and overnight. This experiment also included samples of the

same volume of *T. phagedenis* phylogroup strain T320A as previous, spiked into doubling volumes of undiluted faeces (2X, 4X, 8X, 16X and 32X). After incubation, 20mg of each spiked faecal sample was subjected to DNA extraction using the DNeasy® blood and tissue kit (Qiagen, United Kingdom) according to the manufacturer's instructions. Extracted DNA was stored at -20°C. Positive controls of *T. phagedenis* phylogroup strain T320A from the stock culture ($1.14x10^8$ cells/ml) and *T. phagedenis*-like phylogroup strain T320A in 1X PBS were included in the DNA extractions. Negative controls consisted of faeces, 1X PBS and OTEB 10% (v/v) foetal calf serum (FCS; Gibco, Paisley, UK). The *T. phagedenis* phylogroup specific single step PCR assay was then carried out on samples from each time point.

3.2.4 Faeces spiking for DNA extraction optimisation

Serial ten-fold dilutions (10^0-10^{-5}) of *T. phagedenis* phylogroup strain T320A in oral treponeme enrichment broth (OTEB; Anaerobe Systems, Morgan Hill, CA, USA) were set up from a pooled stock of *T. phagedenis* phylogroup strain T320A cultures diluted to $1.14x10^8$cells/ml (Evans et al., 2009a). A two-fold dilution (1/2) of stock into OTEB was also set up. Several replicates of this dilution series were made for different DNA extraction optimisation experiments and stored at -20°C. Faeces from four different cows (two cows which produce low milk yields and two cows which produce high milk yields) that had previously been shown to be negative using nested PCR assays for DD treponeme phylogroups were pooled and mixed. Then 1 g of faeces was spiked with 100µl of the treponeme dilution series set up previously (up to dilution of 10^{-5}). Spiked faecal samples were vortexed and stirred with a pipette tip to mix. Three replicates of spiked faecal samples for each dilution were made and stored at -20°C.

3.2.5 DNA extraction optimisation

1) DNA was extracted from the *T. phagedenis* phylogroup strain T320A dilution series (no faeces) set up in Section 3.2.6 using five DNA extractions methods/ commercial kits (Table 3.1). DNA extraction was carried out according to the manufacturer's instructions (see Appendix A) for kits DNeasy® Blood and Tissue Kit (QT; Qiagen, Manchester, UK), QIAamp® Fast DNA Stool Mini Kit (QS; Qiagen, Manchester, UK), Wizard® Genomic DNA Purification Kit (PW; Promega, Southampton, UK) and PowerSoil® DNA Isolation Kit (MP; MO BIO laboratories Inc, Carslbad, CA, USA). However, an extra centrifugation step for 3 minutes was added at step 12 in the manufacturer's instructions for the PW kit and all centrifugation steps from step 12 onwards were carried out at 4˚C (Appendix A). Method CB was a crude boiling DNA extraction method (Clegg et al., 2011). Briefly, the sample to be

DNA extracted from was diluted 1 in 10 in PBS and centrifuged at 14 000 *g*. The supernatant (1 ml) was boiled for 15 minutes and then cooled on ice for 20 minutes followed by centrifugation for 5 minutes at 14 000 *g*. The supernatant was kept and stored at -4˚C.

2) Faeces spiked with the serial dilutions of *T. phagedenis* DD phylogroup strain T320A were subjected to the same five DNA extraction method/ commercial kits (Table 3.1) as in experiment 1. Extractions were carried out using the same protocols as above for each kit (Appendix A), except for the PW kit in which an initial ten-fold dilution step of spiked faeces in 1X PBS was added, followed by 2 minute sedimentation before the liquid above the sedimented faeces was taken for use in the following steps of the extraction protocol (Appendix A).

Table 3.1: Five DNA extraction methods/ commercial kits used for faeces DNA extraction optimisation

Abbreviation	Kit/ method name	Manufacturer	Material starting amount	Extraction type	Elution volume (µl)
QT	DNeasy® Blood and Tissue Kit	Qiagen, Manchester, UK	20 mg	chemical	100
QS	QIAamp® Fast DNA Stool Mini Kit	Qiagen, Manchester, UK	200 mg	chemical	200
PW	Wizard® Genomic DNA Purification Kit	Promega, Southampton, UK	1 ml	chemical	100
MP	PowerSoil® DNA Isolation Kit	MO BIO laboratories Inc, Carslbad, CA, USA	250 mg	physical/ chemical	100
CB	Crude Boiling Method	N/A	1.5 ml	physical	1000

In both experiments the positive control was *T. phagedenis* phylogroup strain T320A culture which had been concentrated 4X and for the faeces spiked with *T. phagedenis* phylogroup strain T320A DNA extraction experiments a second positive control of the T320A stock culture used to make the original serial dilution series was also included.

Negative controls included OTEB, water and the pooled faecal samples used for spiking with *T. phagedenis* phylogroup strain T320A.

3.2.6 Evaluation of DNA extraction methods for digital dermatitis treponeme culture and faeces spiked with digital dermatitis treponemes

For both experiments 1 and 2, extracted genomic DNA from each method/ commercial kit was run on a 1% agarose gel containing ethidium bromide in 1X TAE buffer to visualise the presence of genomic DNA and its integrity. *T. phagedenis* phylogroup specific single step PCR assays were carried out as described previously (Section 3.2.1) to detect *T. phagedenis* phylogroup strain T320A DNA within the extracted genomic DNA. The purity of the extracted genomic DNA was measured using a spectrometer to obtain an A_{260}/A_{280} ratio; with pure DNA deemed to be present at ratios between 1.8 and 2.0. Finally the concentration of the extracted DNA was determined using the Qubit® dsDNA BR assay kit with the Qubit® 2.0 Fluorometer (Life Technologies, Paisley, UK).

3.2.7 Faeces spiking for alternative DNA extraction protocols

Laboratory work carried out pertaining to chapter sections 3.2.7-9 were carried out by Jenna Lowe a member of the Infection Biology technical team of the Institute of Infection and Global Health with input and analysis from the author.

Serial two-fold dilutions of *T. phagedenis* phylogroup strain T320A in OTEB (1/2 – 1/1048576) were set up using pooled cultures of T320A containing 1.14 x 10^8 cells/ml. The pooled cultures were also concentrated 2X. Faecal samples from four different cows (low milk yield and high milk yield cows), that had previously been shown to be negative using nested PCR assays specific for DD treponeme phylogroups, were pooled and mixed. Five hundred milligrams of faeces was spiked with 500µl of each dilution from the two-fold *T. phagedenis* phylogroup strain T320A dilutions series as well as with the 2X concentrated T320A. Spiked faecal samples were vortexed and stirred with a pipette tip to mix. Spiked faecal samples were stored at -20°C.

3.2.8 Alternative DNA extraction protocols optimisation

Faeces spiked with an equal volume of two fold serial dilutions of *T. phagedenis* phylogroup strain T320A up to a dilution of 1/8, underwent DNA extraction using two DNA extraction techniques, PowerFecal® DNA Isolation Kit (PF; MO BIO laboratories Inc, California, USA) and Stool DNA isolation Kit (BN; Norgen Biotek Corp, Thorold, Canada) which had not

previously been tested with DD treponeme spiked faeces and two DNA extraction kits that had been used in the previous experiment but with varying protocols (Tables 3.1 and 3.2). Genomic DNA was extracted from the DD treponeme spiked faeces using the standard protocol according to the manufacturer's instructions for all four kits (PF, MP, QS and BN; see Appendix A for full protocols) (Table 3.1 and 3.2), although three different starting quantities of spiked faeces were used for BN (BN.1-3; Table 3.2).

Alternative additional steps were also added to the standard protocols employed for MP and QS (Table 3.2; see Appendix A for full protocols). Genomic DNA extracted by QS underwent variations of the standard protocol in two separate extractions: QS.1) an alternative temperature of 95°C, for difficult to lyse bacteria, was employed for the lysis step in the protocol as suggested by the manufacturer's instructions. QS.2) Pre-treatment of the starting amount of DD treponeme spiked faeces by homogenisation with 1.4ml of ASL Buffer (included in the QIAamp® DNA Stool Mini kit, Qiagen, Manchester, UK) followed by incubation for 10mins at 70°C was carried out (Klitgaard et al., 2014). Following both alternate variations of the standard protocol DNA extraction was completed as stated in the manufacturer's protocol. The variations on the standard protocol used during two separate MP extractions as suggested by the manufacturer were MP.1) the sample was treated as a 'wet soil sample'. This method involved an initial step of centrifuging 0.25 g of the DD treponeme spiked faecal sample in the PowerBead Tubes (MO BIO laboratories Inc, California, USA) provided (removing the PowerBeads and Solution beforehand) at 10 000 x *g* for 30 seconds at room temperature. As much of the supernatant as possible was then discarded. The PowerBeads and Solution were then added back to the PowerBead Tube containing the centrifuged faeces and the protocol was carried out according to the manufacturer's protocol as described in Appendix A. MP.2) the second alternative protocol used with the MP extraction kit changed the lysis step from the original protocol to another that was suggested by the manufacturer for difficult to lyse bacteria. Briefly, after adding Solution C1 as described in the standard protocol the samples were vortexed for 3-4 seconds, followed by heating for 5 minutes at 70°C. The two steps were repeated a second time and followed by a final vortex for 3-4 seconds before following the standard manufacturer's protocol as described in Appendix A.

Table 3.2: DNA extraction commercial kits and protocols used for alternative DNA extraction protocols experiment

Abbreviation	Kit/ method name	Manufacturer	Material starting amount (mg)	Extraction type	Elution volume (µl)
QS.1	QIAamp® Fast DNA Stool Mini Kit – alternative lysis method	Qiagen, Manchester, UK	200	chemical	200
QS.2	QIAamp® Fast DNA Stool Mini Kit – pretreatment protocol (Klitgaard et al., 2014)	Qiagen, Manchester, UK	200	chemical	200
MP.1	PowerSoil® DNA Isolation Kit – wet soil sample protocol	MO BIO laboratories Inc, Carslbad, CA, USA	250	Physical/ chemical	100
MP.2	PowerSoil® DNA Isolation Kit- alternative lysis protocol	MO BIO laboratories Inc, Carslbad, CA, USA	250	Physical/ chemical	100
PF	PowerFecal® DNA Isolation Kit	MO BIO laboratories Inc, Carslbad, CA, USA	250	Physical / chemical	100
BN	Stool DNA isolation Kit	Norgen Biotek Corp, Thorold, Canada	100 (BN.1), 150 (BN.2), 200 (BN.3)	Physical/ chemical	50

Following the results from the above DNA extraction protocols the QS.1 protocol was repeated with faeces spiked with serial two-fold dilutions of *T. phagedenis* phylogroup strain T320A down to a dilution of 1/1048576 in order to determine the extraction methods sensitivity. A large dilution range was carried out to ensure it incorporated the dilution which may be the limit of detection for the extraction. Whilst a two-fold dilution series was chosen so that a more detailed and accurate understanding of the sensitivity of

the extraction method may be obtained than what would be obtained if a ten-fold dilution series was used.

For all extractions carried out above negative controls of OTEB and faeces without *T. phagedenis* phylogroup strain T320A were included. A positive control of T320A stock culture (1.14 x 10^8 cells/ml) used to make the serial two fold dilutions of T320A for spiking the faeces was also included.

3.2.9 Evaluation of DNA extraction methods for alternative DNA extraction protocols

All extracted samples from the alternative DNA extraction protocol experiments were subjected to the *T. phagedenis* phylogroup specific single step PCR assay for the detection of the *T. phagedenis* phylogroup strain T320A DNA within the genomic DNA extracted from the DD treponeme spiked faeces. Extracted samples that were PCR positive for the *T. phagedenis* phylogroup had the DNA concentration analysed using the Qubit® dsDNA BR assay kit with the Qubit® 2.0 fluorometer (Life Technologies, Paisley, UK). The purity of the extracted genomic DNA was analysed using the A_{260}/A_{280} ratio function of the Nanodrop 2000 spectrometer (Thermo Scientific, Hemel Hempstead, UK).

3.3 Results

3.3.1 Optimisation of single step PCR assays specific for each of the three digital dermatitis associated treponeme phylogroups

To enhance DD-associated treponeme detection, the second step of the phylogroup specific nested PCR assays (Evans et al., 2009c) were optimised for use as single step 'non-nested' PCR assays. This change removes potential bias for more abundant bacterial species in bovine faeces created by the initial eubacterial step of the DD treponeme phylogroup specific nested PCR assays. This was achieved by step removal as well as changing the cycle number of the second step of the DD treponeme phylogroup specific nested PCR assays in an attempt to mitigate sensitivity reduction. For each phylogroup: 40, 45, 50 and 55 cycle reaction conditions were investigated for ability to produce unambiguous PCR product bands that remained specific for the target DD treponeme phylogroup. The clearest reproducible PCR product bands, specific to the targeted DD treponeme phylogroup, were obtained using 50 cycles for each of the DD treponeme phylogroup specific single step PCR assays (Figure 3.1a). Below 50 cycles PCR product bands

for the targeted DD treponeme phylogroup were not always produced, and above this number the specificity of the PCR assay was reduced which resulted in bands of the incorrect size and for DD treponemes of a different phylogroup acting as negative controls (Figure 3.1b). Variations in results were obtained between different PCR machines that had differing ramp rates (3°C/s or 6°C/s). The ramp rate refers to the speed in which the PCR machine can change temperature over time, expressed as °C/s. If the PCR programme was altered to the lower ramp rate of 3°C/s on the two PCR machines tested, then the results were comparable between these machines.

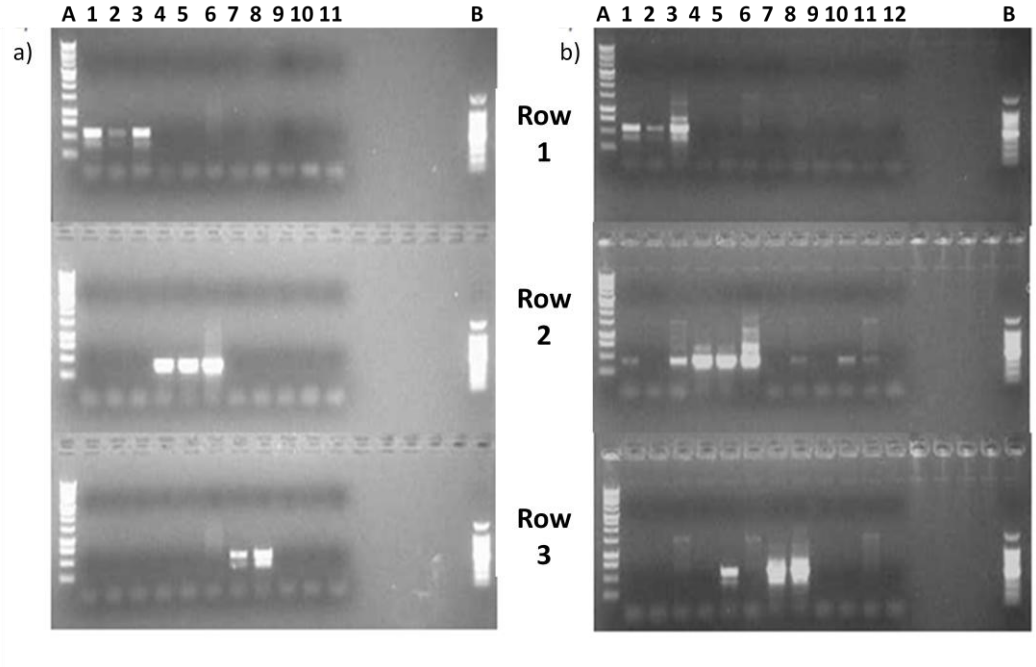


***Figure 3.1: 50 and 55 cycle* single step DD treponeme phylogroup specific *PCR* assay results**

Figure 3.1 a) and b) depict the 50 and 55 cycle single step PCR results for each DD treponeme phylogroup respectively. Row 1 is the T. medium-like phylogroup specific PCR assay results. Row 2 is the T. phagedenis phylogroup specific PCR assay results. Row 3 is the T. pedis phylogroup specific PCR assay results. Figure 3.1 a) lanes 1-3 are the T. medium phylogroup positive controls T19, T56 and T. medium respectively. Lanes 4-6 are the T. phagedenis phylogroup positive controls T320A, T354A and T. phagedenis respectively. Lanes 7 and 8 are the T. pedis phylogroup positive controls T3552B^T and T354B respectively. Lane 9 is the T. denticola negative control, nearest relative to T. pedis. Lane 10 is the commensal treponeme Oc1 and Lane 11 is water. Lane A is the 1 Kb ladder and Lane B is the 100 bp ladder for PCR product band size reference. Figure 3.1 b) Lanes are the same as figure a) except that lane 11 is the commensal treponeme Ru2 and lane 12 is water.

Sensitivity assays using a ten-fold dilution series for each of the three DD treponeme phylogroups (*T. medium* phylogroup strain T19, *T. phagedenis* phylogroup strain T320A and *T. pedis* strain T3552B^{T}) were carried out to determine if the DD treponeme phylogroup specific single step PCR assays had comparable sensitivities to the DD treponeme phylogroup specific nested PCR assays currently used for DD treponeme detection (Evans et al., 2009c). For each single dilution of the series, the DD treponeme specific PCR assay was carried out in triplicate as would normally occur if a sample was being tested for the detection of DD treponemes (Chapter 2 Section 2.7.2). Due to the observed variability in sensitivity per replicate, a median of the results was taken as the overall sensitivity of each assay. Overall for the *T. medium* phylogroup and *T. phagedenis* phylogroup the single step PCR assays had comparable sensitivities to the equivalent nested PCR assays (Table 3.3) with the *T. medium* phylogroup specific nested PCR assay and single step assay both able to detect to a median dilution of 10^{-1} for *T. medium* phylogroup strain T19. The *T. phagedenis* phylogroup specific PCR assays and the *T. pedis* specific PCR assays had slightly different, but comparable sensitivities for *T.* phagedenis phylogroup strain T320A and *T. pedis* strain T3552B^{T} with a sensitivity of 10^{-4} for the nested PCR assay and 10^{-3} for the single step PCR assay. Usually the variability observed between replicates within the DD treponeme phylogroup specific nested and single step PCR assays was within one dilution of each other, for example, 10^{-1} and 10^{-2} for the *T. medium* phylogroup specific nested PCR assays. However, occasionally the difference is larger between sensitivities for the replicates as observed with the *T. pedis* specific nested PCR, with sensitivity results of 10^{-5}, 10^{-4} and 10^{-1} for each replicate.

Table 3.3 Comparison of the sensitivities of the single step phylogroup specific PCR assays and the nested phylogroup specific PCR assays for each of the three DD treponeme phylogroups

		Replicate number[b]			
Treponeme phylogroup	**PCR type[a]**	**1**	**2**	**3**	**Median**
T. medium	Single step	10^{0}	10^{-1}	10^{-1}	10^{-1}
	Nested	10^{-2}	10^{-1}	10^{-1}	10^{-1}
T. phagedenis	Single step	10^{-3}	10^{-3}	10^{-1}	10^{-3}
	Nested	10^{-4}	10^{-3}	10^{-4}	10^{-4}
T. pedis	Single step	10^{-3}	10^{-3}	10^{0}	10^{-3}
	Nested	10^{-5}	10^{-4}	10^{-1}	10^{-4}

[a] *Nested and single step PCR assays specific for each DD treponeme phylogroup.*

[b] *Each PCR assay was carried out in triplicate for each single dilution series and a median result of the replicates given.*

3.3.2 Analysis of the *T. pedis* specific PCR primers

It was noted during processing of samples with the *T. pedis* specific nested PCR assay and for the optimisation of a single step *T. pedis* specific PCR assay (Section 3.3.1), that *T. pedis* strain T354A would always produce a moderate to strong band after gel electrophoresis of the PCR product; whereas *T. pedis* strain T3552B^T produced bands on the gel that would vary greatly in strength or would not be present at all. This apparent trend between the two strains prompted investigation into the *T. pedis* specific primers. A CLUSTALW alignment of the 16S rRNA gene sequences of *T. pedis* strains T354A and T3552B^T was used to determine if there were any base changes in the sequences between these two *T. pedis* strains where the *T. pedis* specific primers annealed. *T. denticola* was also included to ensure the primers were still specific for the *T. pedis* phylogroup only. The aligned sequences were compared against the *T. pedis* specific forward and reverse primers (Chapter 2 Table 2.4). It was discovered that where the forward primer anneals in the *T. pedis* strain T3552B^T 16S rRNA gene sequence, there is a single nucleotide polymorphism (SNP) which was not present in *T. pedis* T354A primer annealing sequence. The forward primer sequence contains a guanidine matching that of *T. pedis* strain T354A (5'-GGAGATGAGGGAATGC**G**TCTTCGATG-3') whereas *T. pedis* stain T3522B^T contains an adenine (5'-GGAGATGAGGGAATGC**A**TCTTCGATG-3') rather than a guanidine in this position of the sequence. Inclusion of *T. denticola* in the alignment confirmed that the primers were specific only to *T. pedis*.

The primer annealing step of the *T. pedis* specific PCR assays was optimised so that both *T. pedis* strains were consistently detected. Initially the annealing time was extended from 30 seconds to 1 minute. However, inconsistencies in the production of a PCR band for *T. pedis* strain T3552B^T persisted. A PCR gradient set up to run +/-5°C of the current 68°C annealing temperature for the *T. pedis* specific single step PCR assay was carried out to determine whether a different annealing temperature could overcome problems in detection of T3552B^T caused by the SNP in the primer annealing sequence. *T. pedis* strains T3552B^T and T354A were subjected to the PCR gradient assay as well as *T. denticola*. An annealing temperature of 67°C was chosen based on the lowest temperature that allowed detection of both *T. pedis* strains but not *T. denticola* (Table 3.4). This temperature was then applied to all *T. pedis* specific PCR assays and was used when determining the sensitivity of the single step *T. pedis* specific PCR assay (Section 3.3.1, Table 3.3).

Table 3.4: Results for the T. pedis phylogroup specific single step PCR assay annealing temperature gradient

	PCR results (+/-)[1]		
Annealing temperature (°C)	***T. pedis* T3552B**	***T. pedis* T354A**	***T. denticola***
63	+	+	+
63.2	+	+	+
63.7	+	+	+
64.7	+	+	+
65.8	+	+	+
67.1	+	+	-
68.5	+	+	-
69.9	+	+	-
71.2	-	+	-
72.2	-	+	-
73	-	-	-
73.5	-	-	-

[1] *(+) denotes the presence of a band after gel electrophoresis of the PCR products and (-) denotes no PCR product band present.*

3.3.3 Faecal inhibition of digital dermatitis treponeme detection assays

Assays were prepared where a set volume of a known amount of *T. phagedenis* phylogroup strain T320A culture was spiked with a set volume of bovine faecal dilutions. Such dilution series should quantify the ability of the optimised single step DD treponeme phylogroup specific PCR assays to detect the DD treponemes in the presence of increasing bovine faeces. In the initial faecal inhibition experiment, incubation of *T. phagedenis* phylogroup strain T320A with the two sets of faecal dilution series (ten-fold and two-fold) was carried out to approximately replicate what would happen during farm sampling: 1 hour at ambient temperature followed by 3 hours on ice. Positive results were obtained for all dilutions except for one of the two *T. phagedenis* phylogroup strain T320A cultures spiked with an equal volume of undiluted faeces (Figure 3.2). These spiked cultures may have been at the limits of detection resulting in inconsistent results for this sample. PCR product band strength did not show a dose dependent increase as would be expected with increasing dilutions of faeces. Although in the two-fold dilution series the bands were slightly weaker for the first three dilutions than the larger dilutions of faeces. A faeces sample that had not been spiked with DD treponemes was negative and therefore proved the bands obtained were from the *T. phagedenis* phylogroup strain T320A culture inoculated into the faecal dilutions and not *T. phagedenis* treponemes naturally present in the faeces.

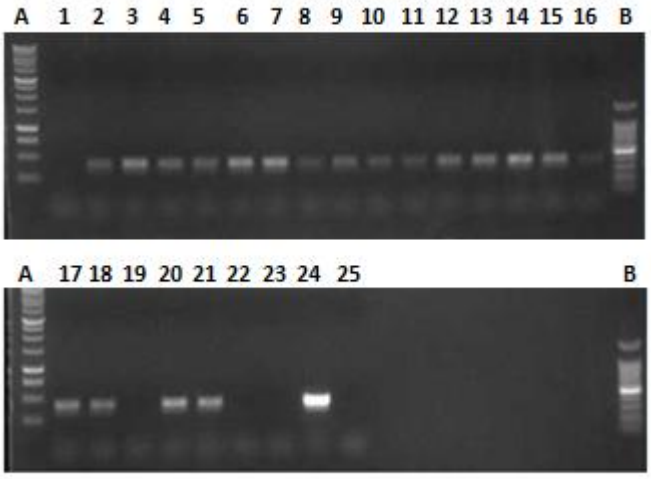


Figure 3.2: Faeces inhibition on DD treponeme detection assay 1

Figure 3.2 shows the gel electrophoresis results from the T. phagedenis phylogroup specific PCR assays following incubation of T. phagedenis phylogroup strain T320A in serial dilutions of faeces at ambient temperature. Lanes 1 and 10 corresponds to samples in which an equal volume of T320A is added to an equal volume of undiluted faecal sample (1:1). Lanes 2-9 are the serial 1 in 10 faecal dilutions (10^{-1}-10^{-8}) added to an equal volume of T320A. Lanes 11-18 are the serial 1 in 2 faecal dilutions (1/2-1/256) added to an equal volume of T320A. Lanes 19, 22 and 23 are the negative controls of faeces, 1X PBS and OTEB 10% FCS without DD treponemes respectively. Lanes 20 and 21 are the positive controls of T320A only and T320A in 1X PBS respectively. Lanes 24 and 25 are the PCR controls of T320A and water.

The second experiment followed a similar format as the previous but only used the two-fold faecal dilution series and also had doubling volumes of undiluted faeces added to the same fixed volume of *T. phagedenis* phylogroup strain T320A. Several incubation times (2 hours, 4 hours, 6 hours and overnight) at ambient temperature were tested to understand what effect incubation length with the faeces may have on the ability of *T. phagedenis* phylogroup strain T320A to be detected by the *T. phagedenis* phylogroup specific PCR assay. For all time points PCR product bands were obtained for all the faecal dilutions (Figure 3.3), except for one dilution (1/8) for the 2 hour time point (Figure 3.3a) which appears to be an anomaly as it was detected after the later incubation times. All incubation times had no PCR product band present for the 16X and 32X increase in volume in faecal samples. There was no dose dependency seen in band strength for 2 hours and 4 hours (Figure 3.3a and b), however, 6 hours and overnight incubations showed increasing band strength with decreasing faecal concentration (Figure 3.3c and d). There did not appear to be a strong relationship between incubation time and detection of *T. phagedenis*

phylogroup strain T320A, although some reduction in detection can be seen between 6 hours and overnight incubations, with the 8X faeces volume increase becoming negative with no PCR product band present and the 4X faeces volume down to the 1:1 samples having weaker PCR product bands.

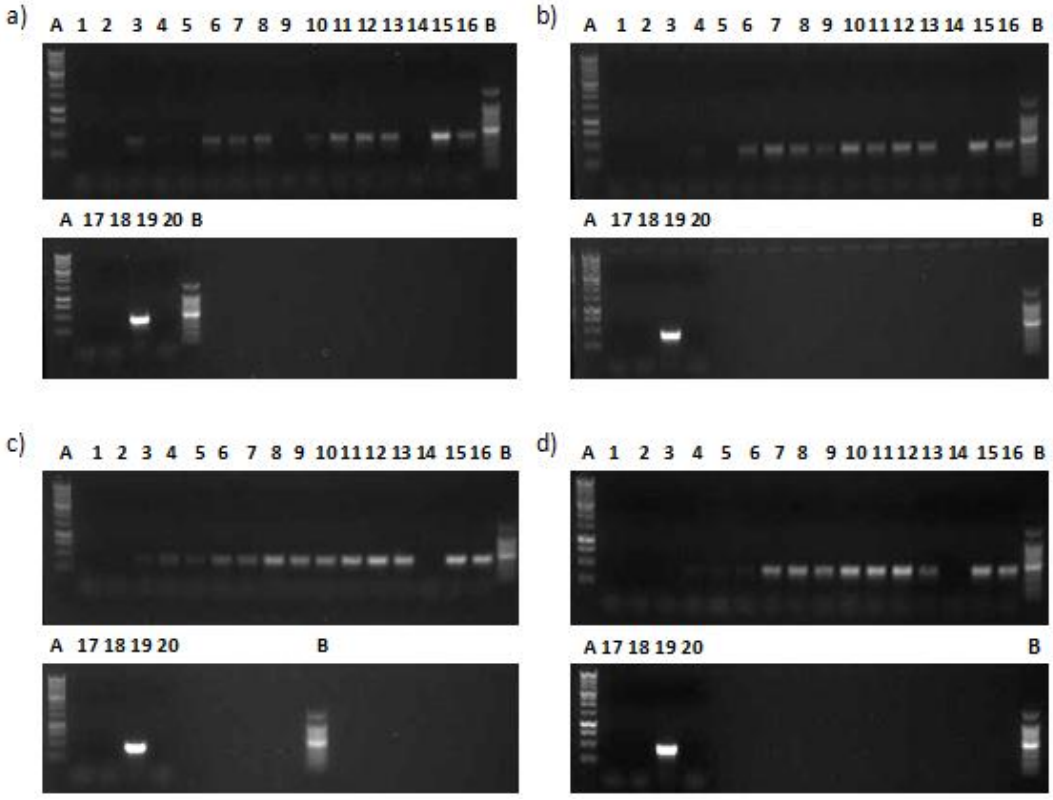


Figure 3.3: Faeces Inhibition on DD treponeme detection assay 2

Figure 3.3 shows the gel electrophoresis results from the T. phagedenis phylogroup specific PCR assays following incubation of T. phagedenis phylogroup strain T320A in varying concentrations of faeces at ambient temperature for a) two hours; b) four hours; c) 6 hours and d) overnight. For each figure a)-d) lanes 1-5 correspond to the doubling volumes of faeces added to T. phagedenis phylogroup strain T320A where lane 1 = 32X volume of faeces, lane 2 = 16X volume of faeces, lane 3 = 8X volume of faeces, lane 4 = 4X volume of faces, lane 5= 2X volume of faeces. Lane 6 corresponds to the sample in which an equal volume of T320A is added to an equal volume of undiluted faecal sample (1:1). Lanes 7-13 depict the serial two fold dilutions of faeces in 1X PBS added to an equal volume of T320A where lane 7 = 1/2 faecal dilution, lane 8 = 1/4 faecal dilution, lane 9 = 1/8 faecal dilution, lane 10 = 1/16 faecal dilution, lane 11 = 1/32 faecal dilution, lane 12 = 1/64 faecal dilution and lane 13 = 1/128 faecal dilution. Lanes 14-18 are extraction controls where lane 14 = negative control of the faecal sample with no T320A added, lane 15 = positive control of stock culture of T320A used in the assay, lane 16 = positive control of T320A stock culture in 1X PBS, lane 17 = negative control of 1X PBS and lane 18 = negative control of OTEB 10% FCS media. Lanes 19 and 20 are PCR controls where lane 19 = T. phagedenis phylogroup strain T320A DNA and lane 20 = water. Lane A = 1Kb DNA ladder and lane B = 100 bp ladder for determining PCR product band size.

3.3.4 DNA extraction optimisation for the detection of digital dermatitis treponemes in culture and in bovine faeces

Initially five extraction methods / commercial kits (QT, QS, PW, MP and CB; Table 3.1) were tested for their ability to extract genomic DNA from ten-fold serial dilutions (10^{0}-10^{-5}) of *T. phagedenis* phylogroup strain T320A ($1.14x10^{8}$ cells/ml) cultures. These DNA extraction kits were chosen based on relevance, commercial availability and in the case of QT, QS and PW on their ability to extract DD-associated treponeme DNA from other types of samples. The integrity of the genomic DNA from the T320A dilutions extracted by each method was analysed by gel electrophoresis; running 10µl of each extracted sample on a 1% agarose gel containing ethidium bromide to allow visualisation of the band of genomic DNA by UV. There was no visible bands present for kits QS, QT, MP and CB. However, bands were visible for kit PW for extracted genomic DNA from the 10^{0} -10^{-1} and 1/2 T320A culture dilutions.

Extracted genomic DNA from each commercial kit/ extraction method underwent a PCR assay for the detection of DNA belonging to the *T. phagedenis* phylogroup. Genomic DNA extracted via kits/ methods QS, PW, MP and CB produced PCR positive results for the detection of *T. phagedenis*-like phylogroup DNA, however, each kit/ method differed as to what dilution of T320A it could detect down to (Table 3.5). Kit PW enabled detection of *T. phagedenis* phylogroup strain T320A from extracted genomic DNA down to the dilution of 10^{-4} followed by kit QS where detection was down to the dilution of 10^{-2}. No T320A genomic DNA could be detected from extractions carried out using kit QT.

DNA yield from extracted dilutions of *T. phagedenis* phylogroup strain T320A, up to the dilution of 10^{-2} or lower depending upon whether T320A was detected by PCR in larger dilutions, was determined via fluorometry (Qubit® fluorometric quantitation). The largest DNA yield per dilution extracted was produced by kit PW (Table 3.5) with the non-diluted (10^{0}) T320A sample yielding 1310 ng of DNA; kit CB followed producing DNA yields of 891, 1030 and 930 ng for samples 10^{0}, 1/2 and 10^{-1}, respectively. However, it must be noted that starting amounts of material varied between kits / methods (Table 3.1) which may have influenced DNA yield obtained. Many of the extracted dilutions produced results which were out of range, meaning that the concentration of DNA in the extracted sample was too low for the fluorometer to detect and therefore a DNA yield could not be obtained. Whilst PW and MP produced lower DNA yields as the samples of *T. phagedenis* phylogroup strain

T320A became more dilute; extraction method CB DNA yield did not appear to depend upon the size of the dilution of T320A (Table 3.5).

The efficiency of the DNA extraction kits/ methods was also measured via the purity of the extracted genomic DNA as expressed via A_{260}/A_{280} ratios. Ratios between 1.7 and 2.0 suggest good quality DNA with a ratio of 1.8 considered 'pure' DNA (Chen et al., 2010). Ratios below these values indicate contaminants in the extracted genomic DNA. Purities were measured for the same extracted *T. phagedenis* phylogroup strain T320A as DNA yield (Table 3.5). Kit PW produced purities of 1.8 and 1.7 for extracted genomic DNA from *T. phagedenis* phylogroup strain dilutions 10^0 and 1/2. A ratio of 1.8 was also obtained for extracted genomic DNA from dilution 10^{-2} with kit MP. Predominantly low purities were obtained for kit QT and method CB. Many of the other kits (e.g. kits QS and MP) produced large A_{260}/A_{280} ratios. Large ratios are often indicative of either RNA contamination or a DNA concentration too low for the sensitivity of the spectrometer used, with the latter more likely due to low DNA concentrations obtained for these kits. There were no bands present indicative of RNA when the extracted genomic DNA samples were analysed for DNA integrity by gel electrophoresis as described earlier. Purity does not appear to be important for downstream detection of *T. phagedenis* phylogroup strain T320A as PCR positive results were not limited to pure extracts.

Faeces spiked ten-fold with serial dilutions of *T. phagedenis* phylogroup strain T320A (10^0-10^{-5} and a 1/2 dilution) underwent DNA extraction with the same five commercial kits/ DNA extraction methods (QT, QS, PW, MP and CB) as the serial dilutions of *T. phagedenis* phylogroup strain T320A culture. Genomic DNA could be visualised on a gel for extraction kits QT, QS, PW and MP but not for method CB, suggesting DNA may be too degraded to visualise (Table 3.6). Bands produced were of a high molecular weight. Smearing could be observed to various degrees for all samples that could be visualised on a gel suggesting possible degradation of DNA for these kits. Faint, smeared low molecular weight bands could be visualised on the gel for genomic DNA extracted by kit QS. This could indicate degraded RNA present in the genomic DNA extracted by kit QS.

Table 3.5: Evaluation of five DNA extraction methods for extraction of DNA from T. phagedenis phylogroup strain T320A serial dilutions

T. phagedenis	DNA yield (ng)					DNA Purity (A_{260}/A_{280})					PCR detection of T320A[a]				
T320A dilution	**QT**	**QS**	**PW**	**MP**	**CB**	**QT**	**QS**	**PW**	**MP**	**CB**	**QT**	**QS**	**PW**	**MP**	**CB**
10^0	16.2	22.6	1310	121	891	1.4	17.5	1.8	2.3	1.3	-	+	+	+	+
1/2	OR	OR	721	63.9	1030	2.5	14	1.7	5.4	1.3	-	+	+	+	-
10^{-1}	OR	OR	124	OR	930	0.5	12.5	1.3	2.7	1.5	-	+	+	-	-
10^{-2}	OR	OR	OR	OR	NT	0.9	10	4	1.8	1.4	-	+	+	-	-
10^{-3}	NT	NT	OR	NT	NT	NT	NT	1	NT	NT	-	-	+	-	-
10^{-4}	NT	NT	OR	NT	NT	NT	NT	4	NT	NT	-	-	+	-	-
10^{-5}	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	-	-	-	-	-

QT, QS, PW, MP and CB refer to commercial DNA extraction kits/ DNA extraction methods detailed in Table 3.1. OR denotes DNA concentration out of range for measurement (too low). NT denotes not tested. (+) denotes a positive result and (-) denotes a negative result.

[a] refers to T. phagedenis phylogroup specific single step PCR assay.

Extracted genomic DNA samples from each *T. phagedenis* phylogroup strain T320A dilution, for each DNA extraction kit/ method, underwent PCR specific for the *T. phagedenis* phylogroup for the detection of T320A. DNA from T320A was not detected in any of the extracted T320A spiked faecal samples, regardless of the DNA extraction kit/ method used. This may in part be due to the DNA degradation observed by gel electrophoresis of the extracted genomic DNA or due to co-purification of inhibitors from the faecal samples. Interestingly, the controls of *T. phagedenis* phylogroup strain T320A stock cultures used to make the dilution series for spiking were detected by the PCR assays but once diluted ten-fold with faeces they were no longer detectable (extracted samples 10^0). Furthermore, the 10^0 samples of T320A used in the *T. phagedenis* phylogroup strain T320A culture DNA extraction optimisation extractions described above are the equivalent of the T320A stock culture control used in this experiment. In the prior experiment, T320A was detected for extracted 10^0 samples using method CB but not QT where as in his experiment detection occurred in QT but not CB.

Table 3.6: Comparison of DNA integrity and detection of T. phagedenis phylogroup strain T320A dilutions spiked ten-fold in faeces between the five different DNA extraction kits/ methods[a]

T. phagedenis	DNA integrity					PCR detection of T320A[b]				
T320A dilution	**QT**	**QS**	**PW**	**MP**	**CB**	**QT**	**QS**	**PW**	**MP**	**CB**
100	+(S)	+(S)	+(S)	+(S)	-	-	-	-	-	-
1/2	+(S)	+(S)	+(S)	+(S)	-	-	-	-	-	-
10^{-1}	+(S)	+(S)	+(S)	+(S)	-	-	-	-	-	-
10^{-2}	+(S)	+(S)	+(S)	+(S)	-	-	-	-	-	-
10^{-3}	+(S)	+(S)	+(S)	+(S)	-	-	-	-	-	-
10^{-4}	+(S)	+(S)	+(S)	+(S)	-	-	-	-	-	-
10^{-5}	+(S)	+(S)	+(S)	+(S)	-	-	-	-	-	-
T320A[c]	+(S)	-	+(S)	+(S)	-	+	+	+	+	-
Faeces[d]	+(S)	+(S)	+(S)	+(S)	-	-	-	-	-	-

[a] QT, QS, PW, MP and CB refer to commercial DNA extraction kits/ DNA extraction methods detailed in Table 3.1. (+) denotes a positive result i.e. presence of a band on a gel and (-) denotes a negative result i.e. no band present on a gel. (S) signifies smearing.

[b] refers to T. phagedenis phylogroup specific single step PCR assay.

[c] T. phagedenis phylogroup strain T320A (1.14 x 10^8cells/ml).

[d] Faeces refers to faeces not spiked with T. phagedenis phylogroup strain T320A

DNA yield of the extracted genomic DNA from *T. phagedenis* phylogroup strain T320A dilutions 10^0-10^{-2} as well as the 1/2 dilution was determined via fluorometry (Qubit® fluorometric quantitation) (Table 3.7). DNA yields were generally between 720 and 912 ng for genomic DNA extracted via kits QT, PW and MP. Starting amounts of material for these

three kits were 20 mg faeces, 1 ml of a 1/10 faecal dilution and 250 mg faeces respectively and elution volumes were all 100 µl (Table 3.1). Genomic DNA extracted by kit QS had much lower DNA yields between 480 and 560 ng whereas DNA yields for method CB were largest ranging between 3050-3330 ng. Starting material amounts for QS and CB were 200 mg faeces and 1.5 ml of a 1/10 dilution of faeces respectively and elution volumes were 200 µl and 1 ml respectively. The high DNA yield produced by CB will have been influenced by its elution volume which is ten-fold larger than for kits QT, PW and MP and five-fold larger than kit QS. On the other hand, despite kit QS having a two-fold larger elution volume than kits QT, PW and MP it had the lowest yield. The starting amount of material did not appear to reflect DNA yield obtained as demonstrated for kits QT, PW and MP which had very different starting amounts. Furthermore, the dilution of *T. phagedenis* phylogroup strain T320A spiked into the faeces did not appear to influence DNA yield.

Purities were analysed via A_{260}/A_{280} ratios for the same extracted dilutions as for DNA yields (Table 3.7). Purities were generally low for genomic DNA extracted by kits/ methods PW, MP and CB suggesting co-purified contaminants. Extracted genomic DNA from kit QT achieved ratios between 1.8 and 2, indicating good quality DNA with ratios closer to 1.8 indicating 'pure' DNA. Genomic DNA extracted by kit QS had abnormally high ratios which could be indicative of RNA contamination. Indeed, gel electrophoresis of the extracted genomic DNA from kit QS showed bands that would be expected from degraded RNA as detailed earlier.

Table 3.7: DNA concentration and DNA purity of extracted genomic DNA from T. phagedenis phylogroup strain T320A dilutions spiked ten-fold in faeces from five different DNA extraction kits/ methods[a]

T. phagedenis	DNA yield (ng)					DNA Purity (A_{260}/A_{280})				
T320A dilution	**QT**	**QS**	**PW**	**MP**	**CB**	**QT**	**QS**	**PW**	**MP**	**CB**
10^0	859	520	835	861	3330	1.8	5	1.2	1.4	1.2
1/2	720	480	808	794	3050	2	5.2	1.3	2	1.2
10^{-1}	753	530	912	889	3060	2	4.5	1.2	1.3	1.3
10^{-2}	771	560	742	912	3200	1.9	4.1	1.2	1.5	1.2
T320A[b]	93.6	70.8	OR	105	1790	3.7	1.1	NT	1.1	1.5
Faeces[c]	772	552	694	925	2650	16	1.5	1.2	0.8	1.3

[a] *QT, QS, PW, MP and CB refer to commercial DNA extraction kits/ DNA extraction methods detailed in Table 3.2. OR denotes DNA concentration out of range for measurement (too low). NT denotes not tested.*

[b] *T. phagedenis phylogroup strain T320A ($1.14x10^8$ cells/ml).*

[c] *Faeces refers to faeces not spiked with T. phagedenis phylogroup strain T320A.*

3.3.5 Alternative DNA extraction protocols optimisation for the detection of digital dermatitis treponemes in bovine faeces

Following the results from the faeces inhibition on DD treponeme detection assays (Section 3.3.3) and the *T. phagedenis* phylogroup strain T320A spiked faeces DNA extraction optimisation experiments (Section 3.3.4), it was hypothesised that the concentration of *T. phagedenis* phylogroup strain T320A may have been too low in the spiked faeces for downstream detection of DD treponemes after DNA extraction with the different DNA extraction kits/ methods. Therefore, to try and increase the chance of DD treponemes being detected in bovine faeces after DNA extraction bovine faecal samples were spiked (1/2) with a two-fold dilution series of *T. phagedenis* phylogroup strain T320A (1-1/8), as well as 2X concentrated T320A and the spiked faeces underwent DNA extraction with kits QS, MP, PF and BN (Table 3.2). Due to a limited quantity of kit PF, only faeces spiked with T320A dilutions 2X – 1/4 were DNA extracted. These kits were chosen based on commercial availability, easiness of use and ability to remove downstream inhibitors present in faeces. In addition, DD treponeme spiked faeces also underwent DNA extraction using altered protocols for kits QS and MP (QS.1, QS.2, MP.1 and MP.2; Table 3.2) to determine if these alternative protocols improved downstream detection of DD-associated treponemes.

Extracted genomic DNA underwent PCR specific for the *T. phagedenis* phylogroup in order to detect the *T. phagedenis* phylogroup strain T320A dilutions spiked into the bovine faeces. The results are depicted in Table 3.8 with band strength of PCR products scored. Kit BN, which was tested using three different starting amounts of material (BN.1-3), failed to detect T320A in any of the spiked faecal samples. Kits QS, MP and PF were all able to detect T320A to varying degrees in the spiked faeces; with the alternative protocols for QS and MP (QS.1, QS.2, MP.1 and MP.2) performing better in terms of downstream detection than the standard protocols for both kits. Overall, kit QS.1 which included an alternate 95°C incubation step during the lysis stage of the manufacturer's protocol was the only DNA extraction kit to produce medium strength bands across all T320A dilutions (2X-1/8); the other kits either weakly detected T320A in faeces with the production of faint bands or bands were initially strong or medium strength and became faint as T320A became more dilute. Interestingly, although detection appeared to be most successful with kit QS.1, this kit was the only one where detection of the undiluted control stock of T320A failed.

Table 3.8: PCR detection of T. phagedenis strain T320A from extracted genomic DNA from four different extraction kits including alternative methods

T. phagedenis	PCR Detection of T320A[b]									
T320A dilution	**QS**	**QS.1**	**QS.2**	**MP**	**MP.1**	**MP.2**	**PF**	**BN.1**	**BN.2**	**BN.3**
2X	++	++	++	+	+	++	+++	-	-	-
1	+	++	++	+	+	+	+	-	-	-
1/2	+	++	+	+	+	+	+	-	-	-
1/4	+	++	+	-	+	+	+	-	-	-
1/8	+	++	+	-	+	+	NT	-	-	-
T320A[c]	+++	-	+++	++	++	+++	+++	++	+++	+++
Faeces[d]	-	-	-	-	-	-	-	-	-	-

[a] *QS, QS.1, QS.2, MP, MP.1, MP.2, PF, BN, BN.1, BN.2 and BN.3 refer to the commercial DNA extraction kits detailed in Table 3.2.*

[b] *T. phagedenis phylogroup specific single step PCR assay. (+) denotes a faint positive PCR product band, (++) denotes a medium strength PCR product band, (+++) denotes a strong PCR product band and (-) denotes no PCR product band present. NT denotes not tested.*

[c] *T. phagedenis phylogroup strain T320A (1.14 x 10^8cells/ml).*

[d] *Faeces refers to faeces not spiked with T. phagedenis phylogroup strain T320A.*

DNA yields of the extracted genomic DNA from *T. phagedenis* phylogroup strain T320A dilution spiked faecal samples for each kit, which allowed detection of T320A downstream, were measured by fluorometry via a Qubit® Fluorometer (Table 3.9). The largest DNA yields of 1364 and 1362 ng were obtained by kit QS (2X concentrated T320A spiked faeces) and QS.1 (undiluted T320A spiked faeces), respectively. This was followed by kit PF with a DNA yield of 1210 ng for the 2X concentrated T320A spike faeces. Incidentally this sample also produced the strongest PCR product band when detecting T320A in the extracted genomic DNA from the spiked faeces. However, DNA yields were approximately ten-fold lower (84.3-119 ng) for all other T320A dilutions spiked in faeces extracted with this kit. Strangely, DNA yields for kit MP and its alternative protocols were much lower, ranging between 49.8 and 174 ng, compared to DNA yields obtained for kit MP in the previous experiment (Section 3.3.4 and Table 3.7) where DNA yields ranged between 794-912 ng. Conversely, DNA yields of genomic DNA extracted by kit QS produced larger yields for the normal manufacturer's protocol for T320A spiked faecal samples (Table 3.9) than in the previous experiment with spiked faeces (Section 3.3.4 and Table 3.7). DNA extraction method QS.1 produced larger DNA yields across the T320A dilution spiked faecal samples than QS.2. On the other hand DNA yields for kits MP.1 and MP.2 were very similar although overall DNA yields were slightly better for kit MP.1. However, DNA yields obtained between these kits and the other kits did not seem to reflect the ability of the kits to allow downstream detection of T320A in faeces.

Purity of the extracted genomic DNA was measured via A_{260}/A_{280} ratios (Table 3.9). A ratio of 1.8 indicates 'pure' DNA with ratios between 1.7 and 2.0 accepted as good quality DNA (Chen et al., 2010), whereas ratios below 1.7 indicate the presence of contaminants. The ratios obtained for kit QS.2 were largely between 1.8 and 2.0 indicating predominantly good purity DNA. Kits QS and QS.1 mainly produced ratios of 2.1 or 2.2 which may suggest good quality DNA but could also point towards potential RNA contamination of the genomic DNA. Kits MP, MP.1, MP.2 and PF all produced purity ratios lower than 1.7 indicating the presence of contaminants, with kit PF not even achieving ratios above 1. Purity of the extracted genomic DNA did not appear to prevent downstream detection of *T. phagedenis* phylogroup strain T320A as T320A could still be detected in genomic DNA with low purities.

Table 3.9: DNA concentration and purity of extracted genomic DNA from 4 different extraction kits including alternative methods[a]

T320A	DNA Yield (ng)							Purity (A_{260}/A_{280})						
Dilution[b]	QS	QS.1	QS.2	MP	MP.1	MP.2	PF	QS	QS.1	QS.2	MP	MP.1	MP.2	PF
2X	1364	1128	406	101	174	106	1210	2.1	2.1	2.0	1.7	1.6	1.5	0.9
1	1060	1362	430	99.2	91.1	109	119	2.1	2.1	2.0	1.6	1.5	1.7	0.2
1/2	1282	1164	534	98.6	123	101	84.3	2.2	1.9	0.7	1.6	1.4	1.5	0.6
1/4	600	884	556	71.8	96	112	99.3	2.2	2.2	1.8	1.7	1.5	1.4	0.3
1/8	910	864	406	49.8	110	85.3	NT	2.1	2.2	2.0	1.4	1.4	1.4	NT

[a] *QS, QS.1, QS.2, MP, MP.1, MP.2 and PF refer to the commercial DNA extraction kits detailed in Table 3.2. NT denotes not tested.*

[b] *T. phagedenis-phylogroup strain T320A dilutions.*

DNA extractions were repeated for kit QS.1 but with a larger range of two-fold serial dilutions of *T. phagedenis* phylogroup strain T320A (1-1/1048576) spiked into an equal volume of bovine faeces to ascertain the largest dilution to which kit QS.1 would enable downstream detection of T320A. Extracted genomic DNA underwent PCR specific for *T. phagedenis*-like phylogroup in order to detect T320A within the faeces. T320A was detectable in bovine faeces down to the 1/512 dilution of T320A, which when the 1/2 dilution of the T320A dilution series into the bovine faeces is taken into account is a final dilution of 1/1024 of T320A in spiked faeces. DNA yields down to the 1/512 dilution of T320A spiked into faeces were between 838 and 1186 ng. Purities ranged from 1.98-2.14 which borders on acceptable DNA purity but suggests a risk of RNA contamination as seen

previously with this method. Both DNA concentration and purity were independent of how dilute T320A was in the faecal sample.

3.4 Discussion

The ability to detect DD-associated treponemes in bovine faeces would provide an important tool for identifying DD infection reservoirs and determining how transmission of these important treponemes may occur. This infection source knowledge can then be applied to control and elimination strategies on farm. The method used to extract DNA from host and environmental samples can influence the performance of downstream detection techniques due to its ability to determine the quantity, purity (i.e. co extraction of inhibitors and RNA) and the quality (level of degradation) of the genomic DNA extracted from a given sample. The animal species the faecal sample originated from is one of the important factors when considering a DNA extraction method. A recent study demonstrated that DNA yield, purity and downstream performance of genomic DNA extracted by different DNA extraction methods varies between faeces from different host species, and certain DNA extraction methods give better results with faeces from certain host species (Hart et al., 2015). The diet of the host species greatly influences the components of the faecal matrix which may affect the efficiency of DNA extraction with certain methods (Tang et al., 2008; Hart et al., 2015). Indeed, bovine faeces is rich in fibre (e.g. lignin, hemicelluloses and cellulose) which can affect DNA extraction efficiency and cause PCR inhibition (Holloway et al., 1981; Monteiro et al., 1997; Wilson, 1997; Harry et al., 1999; Rapp, 2010). Furthermore, the ability of the DNA extraction method to extract genomic DNA from the target bacterial species must also be considered as the structural integrity of some types of bacteria can make them difficult to lyse (Maukonen et al., 2012). Therefore the method used to extract genomic DNA for downstream detection applications must be taken into careful consideration. This study aimed to improve the possibility of detection of DD treponemes in bovine faeces by optimising a DNA extraction technique for this purpose and also ensuring that the PCR assays utilised were optimal for detection of DD treponemes in extracted genomic DNA from bovine faeces.

Previous studies conducted by this laboratory have employed DD treponeme phylogroup specific nested PCR assays to successfully detect DD treponemes in animal tissue samples, but have thus far failed to detect DD treponemes in bovine faecal samples (Evans et al., 2009c, 2012b, Sullivan et al., 2013, 2014b, Clegg et al., 2015, 2016b). Metagenomic studies have successfully detected and analysed the diversity of treponemes in DD lesions, faeces

and slurry using 16S rRNA gene primers specific for a range of DD treponemes (Klitgaard et al., 2014; Nielsen et al., 2016). Optimised single step DD treponeme phylogroup specific PCR assays were developed in order to negate potential bias for bacteria more abundant in bovine faeces than DD treponemes created by the initial universal bacterial 16S rRNA step of the DD treponeme phylogroup specific nested PCR assays (Evans et al., 2009c). The optimised single step DD treponeme PCR assays remained specific for the targeted DD treponeme phylogroups whilst having sensitivities comparable to the equivalent DD treponeme phylogroup specific nested PCR assays. The faecal inhibition assays employed the optimised single step *T. phagedenis* phylogroup specific PCR assay and was successfully able to detect *T. phagedenis* phylogroup strain T320A DNA when spiked with varying dilutions of faeces.

When comparing the optimised PCR assays on different thermocyclers it was discovered that consistent results could only be obtained across machines if ramp rates were the same and set to the lower ramp rate of 3°C/s. The 3°C/s ramp rate may have performed better than the 6°C/s ramp rate as the lower ramp rate gives the PCR reaction enough time over a critical threshold temperature for each step of the reaction to be completed; especially where GC rich sequences are concerned as in the case of DD treponemes (Aird et al., 2011). Further optimisation may be required to allow consistent results to be produced across a range of thermocyclers regardless of ramp speed.

An investigation into issues with detection of *T. pedis* phylogroup strain T3552B^T with the *T. pedis* phylogroup specific PCR assays was carried out. Sequence comparisons revealed an SNP in the sequence of T3552B^T where the *T. pedis* phylogroup specific forward primer annealed resulting in a mismatch. This SNP is not present in the primer sequence or in *T. pedis* phylogroup strain T354A which had no issues with detection by PCR. Differences were observed in cross reactivity across thermocyclers and we consider that older thermocyclers with slower ramp speeds may have reproducibly enabled respective primer binding to *T. pedis* phylogroup strain T3552B^T reported in earlier studies (Evans et al., 2009c, 2012b). However, with the advent of increasing ramp speeds it is important for the *T. pedis* phylogroup specific PCR assays to continue to detect T3552B^T so correct DD treponeme associations can be determined. A gradient PCR was set up with the two *T. pedis* phylogroup strains and their nearest relative, *T. denticola*, to see if changing the annealing temperature would allow consistent detection of T3552B^T without losing specificity. Changing the annealing temperature from 68°C for 30 seconds to 67°C for 1 minute

appeared to resolve this issue and allowed the PCR assay to remain specific for *T. pedis* only. The new annealing temperature and time were then applied to the single step *T. pedis* phylogroup specific PCR assay allowing sensitivity of the assay to be determined and it was applied to the *T. pedis* phylogroup specific nested PCR assays used in the following chapter of this thesis (Chapter 4) to determine the correct DD treponeme phylogroup associations.

Bovine faecal inhibition assays were carried out to investigate the effect of increasing levels of bovine faeces on the detection of DD treponemes in a sample. The initial experiment and incubation times of 2 and 4 hours from the second experiment did not show a dose response relationship in relation to the concentration of bovine faeces present. A dose response would be expected if inhibition was occurring, as a result of levels of inhibitors increasing with increased faecal concentration. However, results from incubation times of 6 hours and overnight did show the expected increase in band intensity when the faeces became increasingly dilute and there was a slight difference between 6 hours and overnight incubations in their ability to detect *T. phagedenis* phylogroup strain T320A with increasing volumes of faeces. Interestingly, the faeces inhibition assays demonstrated that *T. phagedenis* phylogroup strain T320A DNA was detectable at ambient temperature in the presence of faeces, suggesting DD treponemes may survive in faeces for a period of time and be more robust than previously thought.

To identify a suitable technique for the extraction of DD treponeme DNA from bovine faeces for downstream detection five commercial DNA extraction kits/ methods (QT, QS, MP, PW and CB; Table 3.1) were investigated. Each kit was evaluated for the ability to extract genomic DNA from ten-fold serial dilutions of *T. phagedenis* phylogroup strain T320A culture and from faeces spiked ten-fold with the T320A serial dilutions. Detection of T320A DNA via the optimised *T. phagedenis* phylogroup specific single step PCR assay was the most important outcome of the DNA extraction method evaluation as detection of DD treponemes was the overall goal of the optimisation. Kits PW and QS had the best performance in terms of *T. phagedenis* phylogroup detection from pure culture extracts with detection limits down to dilutions of 10^{-4} (1.14×10^4 cells/ ml) and 10^{-2} (1.14×10^6 cells/ml), respectively. However, when the same *T. phagedenis* phylogroup strain T320A serial dilutions were spiked ten-fold with bovine faeces downstream detection of *T. phagedenis* phylogroup DNA failed for all five DNA extraction commercial kits/ methods, despite the high yields of genomic DNA extracted.

An explanation for why detection of T320A in faeces may have failed is because PCR inhibitors present in the faeces may have been co-purified with the genomic DNA (Monteiro et al., 1997). Of the five kits, only two of these kits (QS and MP) contain inhibitor removal technology for the removal of PCR inhibitors present in faecal samples. However, despite the inclusion of the inhibitor removal technologies, detection for these two commercial kits also failed. Another explanation is that once the *T. phagedenis* phylogroup strain T320A dilutions were diluted further by ten-fold inoculation into faeces, the concentration of T320A was too low for downstream detection. This was illustrated with the faecal inhibition assays where two fold increasing volumes of faeces resulted in poor DD treponeme detection in the faeces as T320A became more dilute. One of the studies reporting the presence of DD treponemes in faeces suggested they only make up a small number of the total *Treponema* microbiota in faeces (Klitgaard et al., 2014). Spiking T320A by ten-fold into faeces was originally chosen to try and allow the concentration of T320A within faeces to be high enough to be detected whilst trying to remain relatively reflective of what the normal concentration of DD treponemes may be in faeces in the field. In addition, the genomic DNA extracted from the T320A culture serial dilutions only contained genomic DNA from T320A itself whereas extracted genomic DNA from the spiked faecal samples contains genomic DNA, not only from T320A, but from the faecal microbiome and other cells found in faeces. It could be hypothesised that the large amount of non target DNA from other sources also present in the extracted genomic DNA could have hindered the *T. phagedenis* phylogroup specific primers from coming into contact with the T320A DNA. An inhibition assay similar to the faecal inhibition assays carried out in this study could be designed to determine the ability of the optimised DD treponeme phylogroup specific PCR assays to detect DD treponeme DNA in the presence of increasing concentrations of non target DNA i.e. from another bacterial species (Picard et al., 1992).

Increasing the concentration of *T. phagedenis* phylogroup strain T320A, by spiking faeces with an equal volume of two fold serial dilutions of T320A, allowed detection of T320A with the optimised *T. phagedenis* phylogroup specific single step PCR assay for all but one of the commercial DNA extraction kits investigated. Interestingly, the alternative protocols for kits MP and QS preformed better in terms of downstream detection of T320A then the standard protocols. Methods MP.1 and QS.2 involved pretreatment steps before following the standard manufacturer's protocols for each kit. Kit MP was originally designed for use with soil but can also successfully be used with faeces (Weingarden et al., 2014; Ng-Nguyen et al., 2015; Zinicola et al., 2015b). The kit works optimally with dry samples, and dairy

cattle faeces is usually very wet, even before the addition of DD treponeme culture dilutions; thus MP.1 involved a pre-treatment step that removes excess liquid from the sample. The inclusion of this step increased the detection limit for T320A spiked in faeces compared to the detection limit for kit MP. QS.2 on the other hand involved a pretreatment step successfully used by Klitgaard et al., (2014) for the detection of DD treponemes in faeces via deep sequencing. This pretreatment step involved homogenisation of the faecal sample and an initial incubation step to begin lysis of the bacterial cells. This pretreatment enabled a slight improvement in the levels of detection of T320A when compared to the standard protocol.

However, for both MP and QS the best results, as measured by limits of detection of *T. phagedenis* phylogroup strain T320A and strength of PCR product band, were obtained when an alternative lysis step was utilised (QS.1 and MP.2). The alternative lysis steps in these kits involved increasing the incubation temperature or introducing a heat lysis step in order to enable lysis of difficult to lyse bacteria i.e. gram positive bacteria and can also help reduce DNA shearing caused by the lysis procedure as with method MP.2. It was also noted that other kits that included incubation at a high temperature during lysis were better able to detect T320A by PCR than those that did not. For example, kit PF which included a heat lysis step, was better able to detect T320A than kit MP which does not include a heat lysis step in the standard protocol. Kit PF is a modified version of kit MP for optimal extraction from faecal samples rather than soil. As not all kits that used a heat lysis step and preformed well downstream had cell lysis procedures with a different temperature to be compared to, a direct comparison of results with different cell lysis temperatures would be required for each kit to determine whether it was the temperature of the heat lysis step that was the important factor for treponeme detection downstream or whether differences in chemicals and procedures were more important.

The thick peptidoglycan cell wall, an identifying feature of gram positive bacteria, makes these bacteria difficult to lyse due to the substantial number of strong bonds formed through cross-linking of the peptidoglycan (Rantakokko-Jalava and Jalava, 2002; Mahalanabis et al., 2009). In comparison, the cell wall of gram negative bacteria is composed of a thin layer of peptidoglycan surrounded by an outer membrane and thus is easier to lyse. Bacteria belonging to the Spirochaete phylum (i.e. *Treponema*) are gram negative with a similar cell wall structure to other gram negative bacteria (Schleifer and Kandler, 1972; Joseph et al., 1973), although they are unique through possessing

endoflagellum contained within the periplasm between the protoplasmic cylinder and outer sheath (Chapter 1 Section 1.6.1). Furthermore the membrane biology of *T. pallidum* has been described as similar to gram positive bacteria due to the presence of membrane lipoproteins with similar functions to those found in gram positive bacteria (Becker et al., 1994). Furthermore, several of the spirochaete genera, including treponemes, are susceptible to penicillin (Evans et al., 2009a); typically a feature of gram positive bacteria. The outer membrane of pathogenic spirochaetes is also reported to be atypical with *Treponema pallidum* and *Borrelia burgdorferi* not possessing lipopolysaccharide (LPS) (Porcella and Schwan, 2001) and Leptospira interrogans exhibiting atypical LPS with novel biological and physical properties (de Souza and Koury, 1992).These novel features of treponemal cell wall architecture may explain why using alternative lysis procedures aimed at difficult to lyse bacteria resulted in the best performance in terms of DD treponeme detection. It is also understood that applying heat during cell lysis can improve the effect of surfactants often used in cell lysis buffers (Rapp, 2010). However, kit QS already involves a heat step and the components of the lysis buffer are undisclosed, but lysis conditions are likely to already be optimised for any surfactants present. Regardless of the reason, the results of this study do indicate that thermal lysis is important for DD treponeme DNA extraction from faeces.

A common method used for DNA extraction of difficult to lyse bacteria, which was not investigated in this study, is pretreatment of the sample with muramidase enzymes such as lysozyme and mutanolysin. These enzymes break down peptidoglycan by hydrolysing the glycosidic bonds between the alternating subunits of N-acetylglucosamine and N-acetylmuramic acid that form the glycan backbone of peptidoglycan thus making it a very effective treatment for gram positive cell lysis (Mahalanabis et al., 2009). Utilisation of muramidases in spirochaete DNA extraction is varied; with only a few studies using lysozyme treatment steps (Barbour, 1988; Choi et al., 1994). Muramidases are often used in microbiome studies where the study aims to extract DNA that is representative of the whole microbiome present, not just spirochaetes (Salonen et al., 2010; Zinicola et al., 2015a).

Another technique applied to DNA extraction protocols for difficult to lyse bacteria is bead beating. This mechanical method involves the use of small beads, made from various materials, added to the sample and homogenised at high speed by shaking/ agitation; which results in cells breaking open and releasing DNA. Bead beating enables simultaneous

sample homogenisation and prevents faecal consistency impeding penetration of lysis buffers (Salonen *et al.*, 2010). DNA extraction kits MP, MP.1, MP.2, PF and BN used in this study all incorporated bead beating as part of their cell lysis procedure. Detection of *T. phagedenis* phylogroup strain T320A via PCR occurred to varying degrees for all bead beating kits except kit BN which failed. However, the kits with the alternative steps in the protocols that did not involve bead beating (QS.1 and QS.2) allowed better detection of T320A. The unmodified QS protocol had the same performance for the detection of T320A as the alternative protocol MP.2. The data from this study suggests bead beating techniques employed by these kits are not advantageous over other methods for extraction and detection of DD treponeme DNA from bovine faeces. This outcome could be due to the composition of dairy cattle faeces as a previous study has shown that bead beating methods and a version of QS known as the QIAamp® DNA stool mini kit (Qiagen, Manchester, UK) have comparable abilities in cell lysis and downstream applications when extracting from bacterial cells without faeces present (Li et al., 2003; Rapp, 2010). However, it would be interesting to combine the alternative methods of kit QS with a bead beating step to see if their combination further improved DNA extraction efficiency. Bead beating has been successfully employed for the detection of *Treponema* DNA from various sources including faeces (Bekele et al., 2011; Klitgaard et al., 2014; Zinicola et al., 2015a; b; Liu et al., 2016). Although a drawback is that prolonged bead beating results in DNA shearing and degradation, especially with gram negative bacteria (Rantakokko-Jalava and Jalava, 2002).

DNA yield was measured to help determine the efficiency of the DNA extraction methods. DNA extraction of the original bacterial culture serial dilutions demonstrated that for the most part DNA yields obtained were reflective of bacterial concentration in the sample, with yields decreasing as T320A became less concentrated. However, DNA yields from method CB did not show the same relationship. Furthermore, the relationship with increasing DNA yield and increasing bacterial concentration was no longer observed once the T320A serial dilutions were spiked two fold or ten fold into bovine faecal samples. This is most likely due to the faecal samples not containing completely homogenous starting amounts of microorganisms before spiking.

Method CB produced some of the largest DNA yields when investigated for the ability to extract DNA from *T. phagedenis* phylogroup strain T320A culture serial dilutions and from the faeces spiked ten-fold with T320A ten-fold serial dilutions. These large DNA yields can

be attributed to method CB requiring having the largest elution volume of 1 ml which will have largely impacted the calculated DNA yield. DNA yields produced by the kits (QT, CB, QS, QS.1, QS.2, PW, MP, MP.1, MP.2 and PF) investigated did not alwas appear to be directly related to the quantity of starting material, which ranged from 20 mg (kit QT) to 1 ml (kit PW). Indeed, kits QT, PW and MP produced similar DNA yields to each other when extracting from faeces despite differing starting amounts of 20 mg, 1 ml of a 1/10 faecal dilution and 250 mg respectively, although the elution volumes of these kits were same. Genomic DNA was eluted into 100 µl for most kits except for kit QS which was eluted into 200 µl and method CB which was eluted into 1 ml, as recommended by the manufacturer or method (Clegg et al., 2011). Some studies investigating DNA extraction methods choose to use the same starting amounts of material and elution volumes across all kits investigated to allow direct comparisons between kits (Claassen et al., 2013; Desneux and Pourcher, 2014; Ferrand et al., 2014); irrespective of starting amount recommended by the manufacturer. Many DNA extraction kit protocols state smaller amounts of material can be used with no adjustment of the protocol. In this study the starting amounts of material and elution volumes were not kept the same and were chosen for each kit/ method investigated based upon what was recommended by each manufacturer in order for the kit/ method to work optimally and provide the best chance of enabling detection of T320A downstream, as this was the most important aspect of the study. Furthermore, as there was a very large range in starting material amounts between kits it would have been very difficult to select an appropriate starting amount of material that would guarantee all kits worked optimally and not provide a bias. A few studies have demonstrated that using a smaller amount of material than the manufacturers recommended amount improved the efficiency of the DNA extraction from faeces (Ariefdjohan et al., 2010; Pontiroli et al., 2011; Ferrand et al., 2014). The effect of smaller amounts of starting material could be investigated for kit QS.1 to see if downstream detection of T320A and DNA extraction efficiency could be further improved.

Kits QS and MP were used in both of the DNA extraction optimisation experiments for *T. phagedenis* DD treponeme phylogroup strain T320A spiked faecal samples and it was observed that these kits performed differently between the experiments in terms of DNA yield. Kit QS produced larger DNA yields in the second experiment investigating alternative extraction methods whereas MP produced much lower DNA yields in the second experiment when compared to the first. These differences may have been related to the differences in experiment set up which may have positively or negatively influenced the

extraction DNA yield depending upon the kit. Different volumes of T320A were spiked into the faecal samples which will have resulted in different faecal sample concentrations and the faecal samples were prepared from pooled faeces from different cows between the experiments. These differences will have resulted in different microbial and inhibitor/ DNA contaminant compositions and concentrations between experiments which may have affected DNA yields.

Strangely DNA yield did not appear to affect downstream detection of *T. phagedenis* phylogroup strain T320A. It would be expected that larger DNA yields would increase the likelihood of T320A DNA being represented within the extracted genomic DNA and would therefore improve T320A detection downstream. This effect has been demonstrated in studies determining the best extraction techniques for microbiome studies where DNA extraction kits that produced the best yields also demonstrated greater diversity of bacterial genera in downstream applications (Claassen et al., 2013; Desneux and Pourcher, 2014). Furthermore in agreement with a study by Hart *et al.*, (2015), purity of the extracted genomic DNA also did not appear to indicate whether T320A would be detected by PCR in that sample, regardless of DNA extraction kit used. For example, a very strong positive PCR result was obtained when the ratio was as low as 0.9 for kit PF but ratios of 1.7 or 1.8, which indicate high purity, achieved negative or faint positive PCR product bands for other kits. A low A_{260}/A_{280} ratio would indicate co-purification of contaminants and many contaminants in faeces act as inhibitors for PCR, however as discussed low ratios did not necessarily appear to inhibit PCR assays in this study.

High purity ratios over a ratio of 2 were observed for genomic DNA extracted by some kits, particularly kit QS which demonstrated high purity ratios across all three DNA extraction experiments. RNA contamination of genomic DNA could explain some of these high ratios. In fact, gel electrophoresis examining the integrity of genomic DNA extracted by kit QS from faeces spiked ten-fold with ten-fold serial dilutions of T320A had bands present that could be consistent with degraded RNA. A study by Claassen *et al*., (2013) compared purity of genomic DNA extracted by the QIAamp® DNA stool mini kit (Qiagen, Manchester, UK), a very similar kit to kit QS, before and after RNase A treatment and found that RNase treatment reduced the purity absorbance ratio from above 2 to levels associated with good quality DNA. RNA absorbs at similar wavelengths to DNA so to ensure the results obtained for DNA extraction efficiency in future experiments is purely due to genomic DNA and not

skewed by RNA contamination protocols could be altered to include an RNase A treatment step to remove RNA and an RNase clean up step to remove the enzyme.

Overall, genomic DNA extracted by kit QS.1 had the greatest performance downstream being the only kit to produce medium strength PCR product bands for all *T. phagedenis* phylogroup strain T320A two fold serial dilutions spiked two fold in bovine faeces. Whilst DNA yield and purity are important measures for DNA extraction efficiency, the ability of the DNA extraction method to enable detection of DD treponemes in bovine faeces was deemed the key criterion for selection of a suitable DNA extraction method to be used for investigating bovine faeces as a potential reservoir for DD infection. Kit QS.1 was further assessed with a larger series of two fold serial dilutions of T320A spiked in bovine faeces to determine the limits of detection of T320A with this kit. Kit QS.1 enabled detection of T320A spiked into faeces down to a final dilution of 1/1024 of the original 1.14 x 10^8 cells/ml culture stock diluted in to bovine faeces. DNA yield and purity remained within the similar ranges as previously observed demonstrating the reproducibility of extraction by kit QS.1.

Whilst to the best of the authors knowledge there does not appear to any studies that investigate kit QS for its ability to extract DNA from bovine faeces compared to other commercial DNA extraction kits; there are studies that evaluate and compare the Qiagen QIAamp® DNA mini stool kit with faeces from various species. As described briefly earlier, kit QS is a more streamlined version of the QIAamp® DNA mini stool kit with the InhibitEX buffer replacing the InhibitEX tablet. Studies have demonstrated that the QIAamp® DNA mini stool kit can successfully extract targeted DNA from bovine faeces (Inglis and Kalischuk, 2003; Inglis et al., 2004; Klitgaard et al., 2014). Although in contrast to the current study; studies comparing the QIAamp® DNA mini stool kit to other commercial kits for DNA extraction of bacterial DNA from faeces (of various species) have predominantly found that genomic DNA extracted by this kit does not perform as well as the kit's competitors in terms of DNA extraction efficiency or in downstream applications (Cook and Britt, 2007; Salonen et al., 2010; Ariefdjohan et al., 2010; Pontiroli et al., 2011; Claassen et al., 2013; Ferrand et al., 2014; Desneux and Pourcher, 2014). Interestingly, several of these studies include the alternative heat lysis temperature used with kit QS.1 in the current study, as well as an initial homogenisation step; many of these studies were interested in the whole faecal microbiome (Salonen et al., 2010; Pontiroli et al., 2011; Claassen et al., 2013; Ferrand et al., 2014).

Whilst the results of kit QS.1 are a promising advance in detection methods for DD treponemes, further optimisation of kit QS.1 may be necessary, especially as the current body of evidence for the presence of DD treponemes in faeces suggests they may be in low numbers and shedding may be limited to a few cows in a herd with RAJ carriage (Evans et al., 2012b; Klitgaard et al., 2014; Zinicola et al., 2015b). Potential optimisation of QS.1 to include RNase A treatment, bead beating for cell lysis and smaller starting amounts of material as described in the above sections may further enhance this method.

The current study used very small amounts of bovine faeces (0.5-1 g) to spike with *T. phagedenis* phylogroup strain T320A and once spiked the faecal samples were thoroughly mixed to ensure homogeneity. In reality, faeces to be processed for DD treponeme detection would be collected in much larger amounts (~50 g). Furthermore it is possible that the distribution of DD treponemes is not homogenous throughout the faecal samples, especially when it is considered that as with *E. coli* O157, DD treponemes can be found in the bovine RAJ and *E. coli* O157 is not homogeneous within faeces, being found in the outer surface (Naylor et al., 2003; Pearce et al., 2004; Robinson et al., 2005). Kit QS.2 included an initial step involving the 200 mg starting amount of faeces homogenised in ASL buffer utilised by Klitgaard et al., (2014), however, this method resulted in reduced DNA yields compared to kit QS, which is in contrast to another study which found DNA yields to be improved by including this initial step (Ariefdjohan et al., 2010). On the other hand, to ensure that DD treponemes are likely to be in the faecal sample taken for DNA extraction an initial homogenisation step (e.g. bead beating) with a larger amount of faeces should be considered before the starting amount of faeces is measured out as well as ensuring different parts of the pat are represented in the faecal sample collected on farm. The manufacturer of QS.1 recommends using the protocol for larger volumes of stool when the targeted microorganism is not homogenous throughout the faeces or in low concentrations.

Optimisation can also occur at the later stages of the DNA extraction protocol. In this study many of the commercial DNA extraction kits (QT, QS, MP and PF) utilise silica membrane spin columns for DNA purification. Silica membrane spin columns are generally considered more user-friendly and less time consuming than other methods such as isoproponal precipitation (Kit PW) and phenol-chloroform methods. However, retention of DNA on silica membranes can occur, with one study finding as much as 79% of DNA loaded into the column retained in the silica membrane after elution (Salonen et al., 2010). DNA yields and

detection may have been affected in the study if retention of DNA, especially treponeme DNA, occurred. Desneux and Pourcher (2014) compared two different elution methods for four kits (including kits PF, MP and the QIAamp® DNA mini stool kit) which utilised silica membrane spin columns. The first elution method was the standard protocol for each kit using 100 µl of the elution buffer. This was then compared to the second elution method where the elution step was carried out successively four times with 25 µl of elution buffer each time, resulting in a total of 100 µl of elute. They found that the second elution method resulted in an increase in DNA yield for each kit compared to yields from the first method and reduced the presence of PCR inhibitors. Thus this second elution method could be investigated with kit QS.1 to see if this method could further improve DNA efficiency and detection of DD treponemes.

The study by Klitgaard et al., (2014) which identified DD treponeme DNA in bovine faeces, stored the faeces collected in RNA*later* stabilisation reagent (Qiagen, Manchester, UK). RNA*later* is designed to stabilise RNA and preserve RNA expression profiles in human and animal tissue samples. However, Klitgaard et al., (2014) were interested in DNA in bovine faeces and were successfully able to detect treponemes in faeces with the inclusion of this reagent. Other stabilisation reagents are available which are designed or can be used for DNA including Allprotect Tissue reagent (Qiagen, Manchester, UK), EDTA buffer (Carozzi and Sani, 2013) and OMNIgene.GUT DNA stabilisation kit (DNA Genotek, ON, Canada). A further benefit of DNA stabilisation reagents is the sample can be stored and protected at ambient temperature for a short period of time which is useful for when collecting samples from farms. Different RNA/DNA stabilisation reagents could be compared for use with kit QS.1 to investigate whether this could improve DD treponeme detection as although the faeces inhibition assays showed treponemes could still be detected in faeces after overnight incubation in ambient temperature, detection in samples with higher concentrations of faeces was reduced with fainter PCR product bands.

A common problem with DNA extraction from faecal samples, especially bovine faeces, is the co-extraction of PCR inhibitors. The faecal PCR inhibition assays demonstrated that high concentrations of faeces in a sample could impact DD treponeme detection via PCR, which may be a result of an increased concentration of PCR inhibitors. In the current study the level of PCR inhibition for each kit was not evaluated and although A_{260}/A_{280} purities were measured, this was not a measure of PCR inhibition, only an indication that there may be inhibitors present if the purity was low. PCR inhibition has been measured in several ways

by other studies: 1) Addition of a known amount of plasmid/DNA into the qPCR/PCR reaction mix containing the extracted faecal genomic DNA and compared to a control containing no extracted genomic DNA (Trochimchuk et al., 2003; Pontiroli et al., 2011); 2) Spiking different amounts of extracted faecal genomic DNA into human DNA to be used as a template for the amplification of a targeted sequence of human DNA and comparing the amount of amplification by qPCR to a human DNA only control (Salonen et al., 2010) or 3) subjecting dilutions of extracted faecal genomic DNA to eubacterial 16S rRNA gene PCR assays (Desneux and Pourcher, 2014) or a qPCR assay specific for the targeted microorganism (Cook and Britt, 2007). A PCR inhibition assay could be modified for use with the *T. phagedenis* phylogroup specific single step PCR assay to determine the level of PCR inhibition that may be occurring with kit QS.1.

Should PCR inhibition be occurring with kit QS.1 following investigation, several methods could be investigated for their ability to dampen the effect of PCR inhibitors to ensure DD treponeme detection is not inhibited. Although already discussed as a method of detecting PCR inhibition extracted faecal genomic DNA can be diluted to reduce the concentration of inhibitors present in the template (Trochimchuk et al., 2003; Cook and Britt, 2007; Desneux and Pourcher, 2014), however, if DNA yields are already low before diluting this method may have a negative impact on detection. It has been suggested that nested PCR assays may be useful to counteract the effects of PCR inhibitors as once the PCR product from the initial amplification step has been added to the master mix of the second nested PCR step the PCR inhibitors will have been diluted (Inglis and Kalischuk, 2003). The DD treponeme phylogroup specific single step PCR assays were originally nested PCR assays before optimisation to removed potential universal 16S rRNA gene bias for more abundant bacteria. The importance of PCR inhibitors vs. universal 16S rRNA gene bias may need to be considered should PCR inhibitors be present in extracted genomic DNA from kit QS.1. Rather than diluting PCR inhibitors for amplification, reagents which aim to neutralise the effects of PCR inhibitors can be added to the PCR master mixes. This includes the previously mentioned PCR facilitators (Section 3.1) such as BSA, betaine or polyethylene glycol (Kreader, 1996; Rudi et al., 2004; Rapp, 2010). Interestingly the QIAamp® DNA mini stool kit recommends the use of BSA and a hot start *Taq* polymerase for PCR assays using extracted genomic DNA from faeces.

PCR based methods have been routinely employed by this laboratory for the detection of DD treponemes in host tissue and the environment (Evans et al., 2009c, 2012, Sullivan et

al., 2015b; c, 2014a; b, 2015a, Clegg et al., 2015, 2016a; c; d; e). PCR is a cost effective, fast method of determining the presence of a microorganism in a sample and is particularly useful for the processing of large sample sizes as is often required in epidemiological studies. Studies which have thus far detected DD treponemes in bovine faeces have favoured PCR based 16S rRNA gene sequencing techniques for analysing the microbial community in faeces (Klitgaard et al., 2014; Zinicola et al., 2015b). Deep sequencing techniques as used by Klitgaard et al., (2014) are advantageous in that it allows detection of microorganisms that may comprise <1% of the microbiome present in a sample. However, PCR has its limitations. A recent study, which compared direct reverse-transcribed small subunit rRNA (RT-SSU rRNA) molecule sequencing of the canine oral microbiome with sequencing of the universal 16S rRNA gene amplicon, found that spirochaetes were under-represented in the canine oral microbiome by 16S rRNA PCR based method of sequencing (McDonald et al., 2016). Further investigation demonstrated that spirochaete sequences had mismatches for the universal 16S rRNA gene primers used in the study which will have lead to the under-representation (McDonald et al., 2016). Indeed, a mismatch in the sequence homologous for the *T. pedis* phylogroup specific forward PCR primer in *T. pedis* strain T3552B^{T} has already been observed in this study. Furthermore PCR may be limited by differing amplification efficiencies of genomic DNA templates and primers may not include novel or divergent strains (McDonald et al., 2016). The use of non PCR based methods of DD treponeme detection could be explored to determine the association of DD treponemes with bovine faeces.

In conclusion, DD treponeme *T. medium, T. phagedenis* and *T. pedis* phylogroup specific nested PCR assays were optimised for use with bovine faeces by removing the first eubacterial 16S rRNA gene step of the nested PCR assays and only utilising the second DD treponeme phylogroup specific PCR assay steps by increasing the cycle number of each assay to 50 cyles to create single step DD treponeme phylogroup specific PCR assays. In addition the *T. pedis* phylogroup specific PCR assay was further optimised by decreasing the annealing temperature and increasing the annealing time to ensure that *T. pedis* phylogroup strain T3552B, which contains a previously unidentified SNP where the *T. pedis* forward primer anneals, could be consistently detected by the assay. The optimised *T. phagedenis* phylogroup specific single step PCR assay was successfully used for the detection of *T. phagedenis* DD treponeme phylogroup strain T320A spiked into bovine faeces as part of the faecal inhibition of DD treponeme detection assays. The faecal inhibition of DD treponeme detection assays did not demonstrate the expected dose

response in band strength of weaker bands with increasing faecal concentration if inhibition was occurring until the T320A had been incubated in the faeces at ambient temperature for 6 hours and overnight before extraction. There was a slight reduction in detection of T320A in the samples with larger faecal concentrations following overnight incubation suggesting faecal samples should not be left overnight at ambient temperature before processing as it may affect DD treponeme detection. Of the commercial DNA extraction kits investigated, Kit PW was the most successful for the PCR detection of T320A in culture whereas Kit QS.1 was the most successful for T320A detection in bovine faeces and was able to detect up to 1/1024 dilution of T320A (1.14×10^8 cells/ ml stock culture) in bovine faeces. DNA yield and A_{260}/A_{280} purity did not seem to reflect upon the downstream ability of the PCR assay to detect T320A in the DNA extracts and were therefore not a good indicator for the usefulness of the extraction kits for downstream detection. The inclusion of a high temperature heat lysis step in DNA extraction protocols appeared to be important for the DNA extraction of DD treponemes. Further optimisation of DD treponeme detection techniques for bovine faeces may be necessary to ensure the maximum likelihood of detection, however, for the purposes of this research project kit QS.1 will be used to investigate whether DD treponemes are present in faeces in Chapter 4.

Chapter 4: Survey of the dairy host and farm environment for digital dermatitis treponeme infection reservoirs

4.1 Introduction

Over 40 years on from the first report of DD in dairy cattle in Italy; DD is now endemic in most bovine milk producing countries and manifestations of the disease have been identified in new species including sheep, goats and wild elk (Cheli and Mortellaro, 1974; Dhawi et al., 2005; Sullivan et al., 2013, 2014b; Clegg et al., 2015). However, despite spread of this infectious disease, there is still very little definitive knowledge about how transmission of DD occurs on and between farms. Current control strategies revolve around individual and whole herd treatment and are failing to adequately control DD; enabling the farmer to reduce some disease prevalence but not eliminate DD on farm (Laven and Logue, 2006). Thus with insufficient (and costly) control strategies and the spread of DD into new species, it is imperative that improved control strategies are developed, focusing on preventative rather than reactive measures which could be achieved through knowledge of how DD is transmitted within the dairy industry.

Studies have been conducted to identify risk factors associated with DD infection in order to inform management practices and reduce risk of the disease on farm. Indeed studies demonstrated an increased risk of DD infection with factors relating to the individual cows such as breed, yield and parity; for example, first parity cows have a greater risk of DD infection than multiparous cows (Rodriguez-Lainz et al., 1999; Somers et al., 2005; Holzhauer et al., 2006; Relun et al., 2013a). Furthermore many farm management practices were predisposing risk factors for DD infection including heifer buying policy where farms that bought in replacement heifers were at greater risk of DD as well as housing system used, floor type, access to pasture and associated levels of farm hygiene (Rodríguez-Lainz et al., 1996; Rodriguez-Lainz et al., 1999; Wells et al., 1999; Somers et al., 2003, 2005; Relun et al., 2013a). Risk factors identified for DD give an indication as to what may cause a farm

or individual to become susceptible to DD infection, however, to understand exactly how transmission is occurring the microbial aetiology of the disease must also be examined.

The knowledge that treponemes are substantially involved in DD lesion development (as described in Chapter 1, Section 1.5.2) enables investigations to determine the infection reservoirs of DD towards breaking the transmission cycles of this disease. There are many definitions of what constitutes an infection reservoir but for the purposes of this study an infection reservoir refers to an environment other than the DD lesion itself in which DD treponemes can sufficiently survive and/or multiply and therefore enable subsequent bacterial transmission. By identifying the infection reservoirs of DD treponemes, protocols can be developed to prevent cattle from becoming infected from these known reservoirs and thus reduce the incidence of DD on farms.

Treponemes are notoriously difficult to isolate and study due to their anaerobic fastidious nature (Wyss, 1992). However, development of molecular based methods for detection of DD treponemes means isolation is no longer the only detection means enabling identification in niches where previous isolation attempts may have failed. Indeed studies have begun to investigate different host tissues and the farm environment for the presence of DD treponemes using molecular techniques and from these studies the GI tract has emerged as having a potential role as an infection reservoir (Evans et al., 2012b; Klitgaard et al., 2014; Sullivan et al., 2015a; Zinicola et al., 2015b).

Evans et al., (2012b) carried out a whole cow survey to identify tissues colonised by the DD treponemes other than the lesions themselves. The internal organs (which included organs of the GI tract, kidneys, liver, lungs, pancreas, bladder and spleen) of three DD-affected and three DD-unaffected cows were investigated for the presence of DD treponeme DNA using nested PCR assays specific for each of the three culturable DD treponeme phylogroups (*T. medium, T. phagedenis and T. pedis*) (Evans et al., 2012b). PCR positive results were obtained from the gingiva adjacent to the upper molar, rumen dorsal sac and rumen at the reticular pillar from one DD-affected cow (Evans et al., 2012b). DD treponemes were also detected in the rectal-anal junction (RAJ) from one DD-unaffected control cow (Evans et al., 2012b). The presence of DD treponemes in the gingiva and rectal tissues were further investigated by sampling multiple sites from the gingiva of eight cows and rectal tissue of 21 cows of varying DD status (Evans et al., 2012b). In total DD treponeme DNA was detected in the gingiva of 14.3% of cattle and the rectal tissue of 14.8% of cattle analysed (Evans et al., 2012b). Positive results were only obtained from gingiva samples taken

adjacent to the upper molar and predominantly from the samples taken from the RAJ although DD treponemes were detected in tissue taken from the rectal wall of one animal (Evans et al., 2012b). Interestingly, there appeared to be an association between detection of DD treponemes in these tissue types and the housing season (October-March) with presence of the *T. phagedenis* DD treponeme phylogroup in the rectal tissue in the housing season proving statistically significant (Evans et al., 2012b).

Additional studies have lead to further confirmation of the GI tract tissues as a potential infection reservoir of DD treponemes with identification of the DD treponemes in rumen fluid of dairy cattle (Nascimento et al., 2015; Zinicola et al., 2015b). Nascimento et al., (2015) discovered that 60% of rumen fluid sampled were PCR positive for one or more of the three cultivable DD treponeme phylogroups (*T. medium*, *T. phagedenis* and *T. pedis*) and positive results were only obtained from DD-affected cows. Zinicola et al., (2015b) investigated rumen fluid from 8 dairy cows for the presence of DD treponemes via shotgun metagenomic sequencing and discovered that rumen fluid had a similar *Treponema* spp. profile to 'active' DD lesions (defined in this study as ulcerative and chronic ulcerative lesions) which includes the three DD treponeme phylogroups commonly isolated from DD lesions in the UK. Rumen fluid may enable transmission due to leakage from the mouth of cows during rumination (Nascimento et al., 2015). However, further investigation into the role of rumen fluid as an infection reservoir would be required.

The knowledge that DD treponemes may be found in the gingiva and RAJ of dairy cattle was applied in a recent infection reservoir survey to beef cattle who also suffer from DD and sheep who suffer from a manifestation of DD known as contagious ovine digital dermatitis (CODD) (Sullivan et al., 2015a). Gingiva and RAJs were sampled from a single site in 40 sheep and beef cattle for the presence of the three cultivable DD treponeme phylogroups (*T. medium*, *T. phagedenis* and *T. pedis*) (Sullivan et al., 2015a). With regard to sheep gingival and RAJ tissues 2.5% and 7.5% were positive for the DD treponeme DNA, respectively (Sullivan et al., 2015a). In beef cattle, 10% of gingival tissues were positive for DD treponeme DNA however, all RAJs were negative for DD treponeme DNA (Sullivan et al., 2015a). In contrast to data from dairy cows, all DD treponeme positive samples were obtained from beef cows and sheep sampled in summer (June-August) (Sullivan et al., 2015a). Furthermore, in a first for ruminants, a treponeme which shared 100% 16S rRNA gene sequence identity with *T. phagedenis* phylogroup strain T320A was isolated from a

sheep RAJ sample, giving evidence that DD treponemes colonising the RAJ are likely to be alive and thus possibly transmissible (Sullivan et al., 2015a).

Colonisation of the RAJ by DD treponemes suggests that shedding of the DD treponemes in faeces may occur, which would enable the DD treponemes from the RAJ to come into contact with the hind feet. Indeed, *Escherichia coli* O157 is an example of a pathogen which colonises the RAJ of cattle and is shed into faeces which acts as a vehicle for transmission (Naylor et al., 2003). Studies utilising PCR detection methods have thus far failed to detect DD treponemes in bovine or ovine faeces (Evans et al., 2012b; Sullivan et al., 2015a). However, recent studies opting for a high throughput sequencing approach successfully detected very small amounts of DD treponeme DNA in faeces from dairy cattle with one study finding on average 0.22% of sequences from faeces were homologous to DD treponeme sequences (Klitgaard et al., 2014; Zinicola et al., 2015b). Slurry, which is a mixture of faeces, urine, bedding and other cattle secretions, has also been shown to contain a very small amount of DD treponeme DNA (up to 0.6% of treponeme sequences) using high throughput sequencing in cows from farms endemic for DD or of unknown status only (Klitgaard et al., 2014, 2017). Furthermore, there was a significant association of DD treponemes with DD status (Klitgaard et al., 2017). The source of the DD treponemes in faeces can only be through the GI tract, however, DD treponemes in slurry could be from other cattle secretions instead, other environmental components of slurry or even from slurry contact with the DD lesions (Klitgaard et al., 2017).

Following the identification of DD treponemes in the gingiva, RAJ and faeces; an avenue which has not been explored yet is the association of DD treponemes with mucin and in particular mucin casts. The gene, *mglB*, which encodes a protein involved in mucin utilisation, has been identified in oral treponemes (Becker et al., 1994) and more recently in DD treponemes (unpublished genome data). It could be hypothesised that DD treponemes may colonise areas where mucin is available, which may explain why they have been detected at the gingiva and RAJ. Furthermore mucin casts found in faeces are a side effect of hind gut acidosis, a condition common in high yielding dairy cows (Gressley et al., 2011; Lean et al., 2013). If DD treponemes are associated with mucin, it may be that they are shed in higher numbers in faeces containing mucin casts. Consequently it would be interesting to investigate mucin casts shed in faeces for the presence of DD treponemes.

Healthy foot skin, with no DD lesion present, is often used as a control for DD studies investigating the microbial aetiology of the disease, with the expected result of DD

treponemes present in lesions and not in healthy foot tissue. Indeed, recent studies investigating the microbiome of DD lesions compared to healthy foot skin using high-throughput sequencing show they have differing microbial profiles with treponemal species the most abundant bacteria in DD lesions, differing vastly to healthy skin (Zinicola et al., 2015a; b; Nielsen et al., 2016). However, unusually, two studies have detected DD treponemes in healthy foot tissue from dairy cows using PCR methodology (Evans et al., 2009c, 2012b). In one study, healthy foot skin from seven DD-unaffected dairy cows from a DD endemic herd were tested for each of the three cultivable DD treponeme phylogroups and DNA from all three DD treponeme phylogroups was detected in healthy foot tissue from one cow (Evans et al., 2009c). In the DD infection reservoir study conducted by Evans et al., (2012b), healthy foot tissue from both the hind and forefeet of three DD-affected and DD-unaffected cows were analysed for the presence of DD treponeme DNA. DD treponeme DNA from one or more of the three DD treponeme phylogroups was detected in at least one type of healthy foot tissue from all three DD-affected cows surveyed (Evans et al., 2012b). This data presents an interesting question: can DD treponemes be present in healthy foot tissue without causing disease? Further investigation is required to understand whether this is actually the case or whether this represents early acute lesions or a type of dormancy.

In addition to faeces and slurry, DD treponemes have recently been identified in another area of the farm environment unrelated to the GI tract. Following the identification of foot trimming as a possible risk factor for DD (Wells et al., 1999), a study investigated the possibility of foot trimming equipment as an infection reservoir of DD treponemes for CODD and DD in sheep and beef cattle respectively (Sullivan et al., 2014a). The blades of foot trimming knives were tested for the three cultivable DD treponeme phylogroups (*T. medium*, *T. phagedenis* and *T. pedis*) following trimming cattle and sheep and overall 62%, 57% and 54% of the 37 blades tested were positive for the *T. medium*, *T. phagedenis* and *T. pedis* DD treponeme phylogroups, respectively (Sullivan et al., 2014a). Additionally a DD treponeme homologous to *T. phagedenis* DD treponeme phylogroup strain T320A was isolated from a blade used on a DD positive animal, providing evidence treponemes may be alive on the blades and thus transmissible (Sullivan et al., 2014a). Trimming blades were also disinfected with a DEFRA approved 2.5% (w/v) available iodine disinfectant (product name not specified) after each use and retested for the presence of the DD treponeme phylogroups; which resulted in a reduction in detection to 24%, 16% and 8% for *T. medium*, *T. phagedenis* and *T. pedis* DD treponeme phylogroups respectively (Sullivan et al., 2014a).

In addition, DD treponemes have also recently been detected and are able to survive for up to three days on gloves, as determined by culture and PCR methodologies, following the handling of cases of CODD in sheep (Angell et al., 2017). A PCR detection rate of 100% on gloves used to handle CODD cases was observed and no detection on gloves used to handle control (CODD-unaffected) sheep. Additionally, washing gloves with either warm or cold water, water and hand soap, 1% (w/v) Virkon solution, a 1:90 dilution of FAM or 70% ethanol was investigated for their ability to disinfect gloves of DD treponemes. DD treponemes were not detected on any of the gloves by culture when washed with either the 1:90 dilution of FAM, 70% ethanol, 1% Virkon solution or water and hand soap suggesting these methods may negatively affect the viability of DD treponemes on gloves. On the other hand, DD treponemes could still be detected on one glove by PCR when 1% Virkon solution or water and hand soap was used. Washing with just cold or warm water was deemed completely ineffective at disinfecting gloves of DD treponemes as DD treponemes were still detected by culture and PCR on all gloves investigated with this method.

The presence of DD treponemes on foot trimming knives and gloves explains the aforementioned association Wells et al., (1999) observed between foot trimming practices and DD infection incidence. Consequently, this data demonstrates a potential transmission route between animals on farm and possibly between farms which must be addressed; highlighting the need to disinfect equipment with a suitable disinfectant which will remove detection of viable DD treponemes. Further work will be required to determine the best disinfection method for all circumstances. Additionally, following the high rate of detection of DD treponemes on gloves used to handle CODD cases it would be prudent to determine if DD treponemes can also be detected on gloves following the handling of DD cases in cattle and whether detection is as high as with CODD. The adherence of DD treponemes to metal is corroborated by studies that identified treponemes involved in human periodontal disease on metallic orthodontic brackets (Nelson-Filho et al., 2011; Andrucioli et al., 2012). This raises the question as to whether other metallic surfaces that come into contact with cattle feet harbour DD treponemes and thus enable transmission through contact and whether only metallic surfaces enable DD treponeme adherence.

The aims of this study are to 1) to build upon current understanding of the association of DD treponemes with the gingiva, RAJ and healthy foot tissue of dairy cattle by surveying a

larger number of these tissues than in previous studies for the presence of DD treponemes by molecular and isolation methods and determining any seasonal variations in DD treponeme association with these tissues. 2) To further investigate the role of the GI tract as an infection reservoir for DD and the presence of DD treponemes in the environment by surveying faeces, mucin casts, feed and water for the detection of DD treponemes by molecular and isolation techniques and 3) To investigate whether other surfaces cattle feet come into contact with might act as an infection reservoir for DD treponemes including gloves, foot trimming equipment (other than the foot knife blades) and hind footprints left on crush and parlour floor surfaces where cattle may stand for a period of time.

4.2 Materials and methods

4.2.1 Farm information

The details of the farms from which environment samples (faeces, mucin casts, feed, water, foot trimming equipment and footprints) were collected are listed in Table 2.3 (Chapter 2 Section 2.3). All farms were DD endemic.

4.2.2 Infection reservoir sample collection

Dairy cattle tissue samples were collected once or twice per month for a period of two years, from a local disposal service for fallen stock serving Lancashire, Cheshire and South Cumbria resulting in 123 cows surveyed. Tissue samples were collected post mortem from dairy cows that had recently been shot within 8 hours of sampling. DD status, breed and tag number were recorded for each cow where possible. Cows were classified as DD-affected if a lesion corresponding to one of the stages of DD (M1-M4.1) described in Chapter 1, Section 1.2.3 was present on one or more of the hind feet. Cows were classified as DD negative (or unaffected) if skin appeared healthy in the area where a lesion would be typically found if the cow was DD-affected. A subset of samples was classified as 'other' where there were abnormalities present on the skin of the feet which could not be definitively classified as a DD lesion. Abnormalities noted included skin abrasions, the skin appearing very dry, rough and 'scaly', what appeared to be excessive skin growth and the presence of thick crust-like scabs not typical of a M3 stage DD lesion scab that would be covering a lesion when healing. No photographs were taken of samples.

Sterile scalpels were used to extract single tissue biopsies from the RAJ (*n*=121), gingiva (gum between the upper first and second premolars) (*n*=122), the skin above the coronet between the bulbs of the heel on both hind limbs (Figure 4.1) for healthy foot tissue

(*n*=217), DD lesional tissue (*n*=12) and foot tissue classed as 'other' (*n*=16). Extracted biopsies were approximately 3 cm^3 in size except for gingival tissue which varied in size (1-2 cm^3) depending upon the conformation of the gum between the upper first and second premolars. Tissue samples were washed in 1X PBS (pH 7.4) if necessary to remove excess blood and dirt. Approximately 3mm^3 of tissue for each sample was transferred into 10% neutral buffered formalin (NBF) for immunohistochemistry (IHC). Another small piece (~5mm^3) of each tissue was placed in transport medium (Chapter 2, Table 2.1) for subsequent culturing. The remainder of the tissue was stored at -20˚C for subsequent PCR analysis. Gloves and scalpels were changed between each sample collected. Samples were kept on ice for transportation. No photographs were taken of samples.

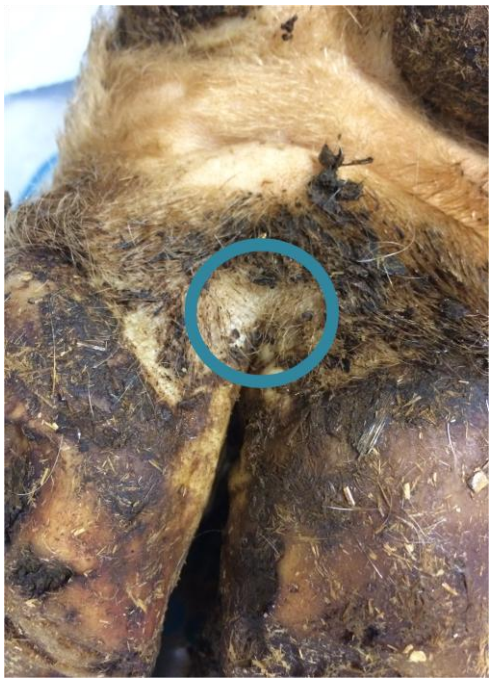


Figure 4.1: Area of healthy foot pedal tissue sampled from the hind foot

Healthy foot and 'other' foot pedal tissues were sampled from the plantar aspect of the hind foot, between the bulbs of the heel as marked by the blue circle. DD lesions were also typically located in this area. Photograph taken before the removal of excess dirt.

A total of 62 faecal samples were collected from two dairy farms (A and B, Chapter 2 Table 2.3) where DD was endemic. The total sample number includes one sample collected previously by another student for the project. Fresh faecal samples were collected immediately after defecation, either by collecting the faeces as it fell during defecation or

by taking a sample from the top layer of the faeces once it had fallen to the ground, in order to avoid cross contamination with the floor. A couple of faecal samples were from dairy cattle of unknown DD status (*n*=4). However, all other samples were from dairy cattle that had their feet lifted and checked for DD by an attending veterinary surgeon at the time of collection or by checking farm records for DD status of the cow following recent checks. Approximately 30-50 g of faeces was collected per sample. For half of the faecal samples (*n*=31), 1 g was transferred into transport medium (Chapter 2, Table 2.1) for isolation by culture. The remainder of the sample was stored at -20˚C for subsequent PCR analysis. Gloves were changed between samples. All samples were transported on ice.

Mucin casts were sampled from faecal samples in which a large amount of faeces (>50g) had initially been collected. A total of 31 mucin casts were collected from one farm (A, Chapter 2 Table 2.3), with 14 of the samples collected previously by another student for this project. To obtain mucin casts, samples of faeces were placed into a sieve (Figure 4.2a) and flushed gently with water until mucin casts were visible as clay-like clumps (Figure 4.2b). Approximately half of the mucin casts (*n*=16) were placed into transport medium using 5 µl inoculation loops three times for subsequent culture. Gloves were changed between samples. Samples were transported on ice and stored at -20˚C for subsequent PCR analysis.

Feed samples were collected from one farm (A, Chapter 2 Table 2.3) and from samples provided by University of Liverpool Veterinary School. Twenty samples of mixed ration feed from the farm were taken over several visits using freshly gloved hands from the top, centre and bottom of feed piles that were provided for the dairy cattle. Where in the feed pile the sample was from and if known whether it was from the high yield, low yield, young stock or the dry cattle barn was recorded. A small proportion of the samples were split (*n*=4) with half immediately stored at -20°C and the other half left to spoil in a falcon tube under ambient conditions for 3 months, after which the spoiled samples were stored at -20°C. The rest of the feed samples were stored at -20˚C. Feed samples provided by the University of the Liverpool Veterinary School were fresh from storage and consisted of 16 different components of feed. All of the university feed samples were transferred directly into transport medium (Chapter 2, Table 2.3) for subsequent culturing. The remainder of the sample was stored at -20°C for subsequent PCR analysis. All feed samples were transported to the laboratory on ice.

a) b)

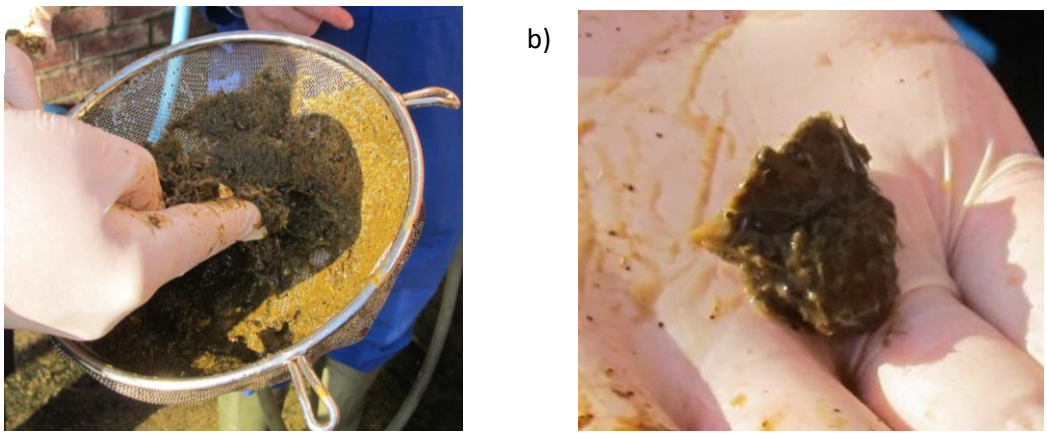


Figure 4.2: mucin cast collection

Figure a) depicts the sieving technique used to obtain mucin casts from faeces. b) depicts an example of a mucin cast. Photograph credit: Rebecca McKown and Dr Richard Murray (University of Liverpool, Liverpool UK).

Water samples (*n*=19) were taken using sterile universals from troughs in the housing areas from two farms (A and F, Chapter 2 Table 2.3). Water samples were transported on ice and stored at -20˚C for subsequent PCR analysis.

Hoof trimming equipment (hoof grinder disc and handle, gloves before and after use and clippers) was swabbed during routine visitation by two different hoof trimmers; foot trimmer one on two different farms (B and C, Chapter 2 Table 2.3) and foot trimmer two on a third farm (E, Chapter 2 Table 2.3). Sampling took place during the foot trimming of cows that had been already chosen by the farmer to undergo their regular foot trimming. The trimmers was allowed to go about their normal foot trimming routine without interference, except to allow a swab to be taken of the equipment, for the floor to be washed between each cow with water and for fresh gloves to be put on between each cow (foot trimmer two only). For each cow the attending veterinary surgeon and foot trimmer examined the hind feet for DD as well as any other hoof disorders and treatment was applied as necessary. Swabs of the hoof grinder disc (*n*= 24) were taken by running a sterile cotton swab (Copan Italia, BS, Italy) back and forth over the surface of the disc before the foot trimming session began and after use on each cow's hind foot. The same method was also applied to the hoof grinder handle (Figure 4.3b) (*n*=19). The part of the clippers (*n*=20) that were in contact with the hoof (Figure 4.3a) was swabbed using the same method as the hoof grinder. Following use on each cow the clippers were washed in cold water and

reswabbed (*n*=18). The gloves worn by the foot trimmer were swabbed when first put on before each cow (*n*=10) and then again after use on each cow (*n*=8). Gloves were swabbed by running the swab back and forth over the fingers and palm of the trimmer's right hand. Further PCR data for gloves swabbed before use (*n*=1) and after use (*n*=8) were kindly donated by a former PhD student. This donated gloves data was collected and processed (DNA extraction and PCR) using the exact same methodology as the gloves samples collected here.

DD status and freeze brand number for each cow was recorded. Swabs were kept on ice for transportation and stored at -20˚C for subsequent PCR analysis.

a) b)

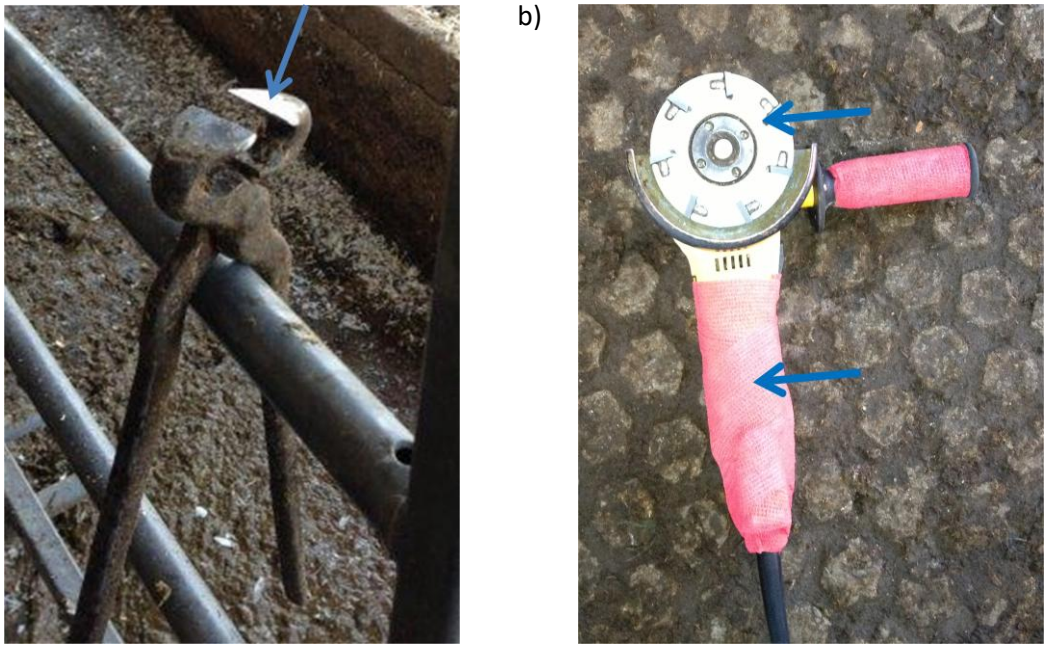


Figure 4.3: Trimming equipment

a) Photograph of clippers used during trimming. Arrow points to edge of clipper where swab is taken of both sides for sampling. b) Photograph of hoof grinder used during trimming. Arrows point to the disc and handle that was sampled by swabbing. Handle is covered in a red cohesive bandage.

Footprints left behind on three types of material used on floor surfaces (rubber *n*=72, concrete *n*=53 and metal *n*=52) were sampled through swabbing footprints on rubber (Farms B, C and E, Chapter 2 Table 2.3) and metal crush floors (Farm A, Chapter 2 Table 2.3) as well as footprints on rubber (Farms B and D, Chapter 2 Table 2.3) and concrete parlour floors (Farm C, Chapter 2 Table 2.3) (Figure 4.4). In the parlour cows were examined for DD

lesions by the attending veterinary surgeon (Figure 4.5), the cow was then encouraged to move its foot forward and a swab was taken of the floor where the foot had just been (the footprint) by running the sterile cotton swab (Copan Italia, BS, Italy) back and forth over the area. Swabs of hoof prints left on crush floors were taken using the same method. However, feet were lifted by a veterinary surgeon or foot trimmer and checked for DD lesions. For each cow, DD status, floor type and freeze brands were recorded. Floor surfaces were regularly washed (between each cow if possible as in the case of crush floors) and swabs of the floor surface taken where the hind feet would normally stand. Swabs were kept on ice for transportation and stored at -20°C for subsequent PCR analysis.

4.2.3 Inoculation of samples into liquid media and subsequent isolation of spirochaetes

Inoculation of tissue and environmental samples for the isolation of spirochaetes, specifically treponemes, was carried out in accordance with culture techniques developed by Evans et al., (2008) for the isolation of treponemes.

Samples in transport medium were transferred into an anaerobic cabinet (85% N_2, 10% H_2 and 5% CO_2, 36 °C) for inoculation. Gingival tissue (*n*=113), RAJ tissue (*n*=115), lesional tissue (*n*=11), healthy foot tissue (*n*=204), 'other' foot tissue (*n*=16) and feed samples (n=16) were decanted from the transport medium into sterile petri-dishes where they were cut into smaller pieces (~1mm^3) using a sterile scalpel blade and inoculated into OTEB supplemented with 10% (v/v) FCS, 25µg/ml rifampicin and 5 µg/ml enrofloxacin (Chapter 2, Table 2.1). For each faecal (*n*=31) and mucin cast sample (*n*=16) in transport medium, three 5µl loopfuls were inoculated into OTEB supplemented with 10% (v/v) FCS, 25µg/ml rifampicin and 5 µg/ml enrofloxacin (Chapter 2, Table 2.1).

Inoculated cultures were checked every 2-3 days during incubation using phase contrast microscopy for the presence of spirochaetes (Chapter 2, Section 2.5.1 and 2.5.3). If spirochaetes were present the culture was sub-cultured onto FAA plates with the addition of 5% (v/v) defribinated sheep blood, 10% (v/v) FCS, 20 µg/ml rifampicin and 5 µg/ml enrofloxacin (Chapter 2, Table 2.1) for single colonies as described in Chapter 2, Section 2.5.2 and 2.5.4. Single colonies were inoculated into OTEB containing 10% (v/v) FCS or RS (Chapter 2, Table 2.1). Phase contrast microscopy was used to ensure a pure treponeme culture was obtained. After sufficient growth, 1.5 ml aliquots of the culture were taken for 16S rRNA gene sequencing. Cultures containing isolated spirochaetes were stored at -80°C in 10% glycerol as described in Chapter 2, Section 2.5.5. For some samples, complete isolation was not possible due to the level of other contaminating bacteria in the culture.

These samples were also stored in glycerol at -80˚C and taken for sequencing if there was sufficient spirochaete growth.

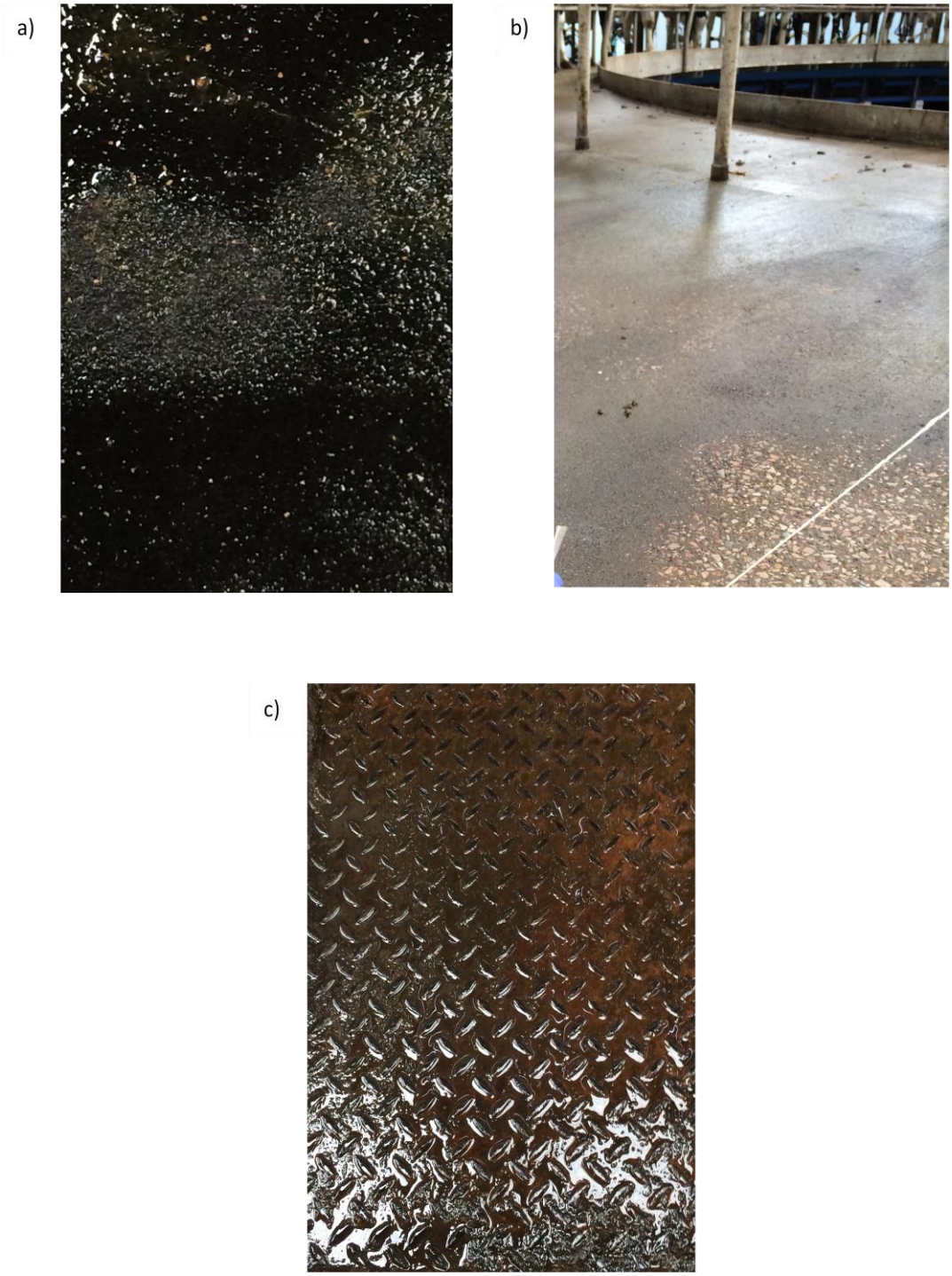


Figure 4.4: Floor surfaces from which footprints were sampled during survey

a) Rubber floor surface of a milking parlour. b) Concrete rotary milking parlour floor surface. c) Metal crush floor surface following washing.

a) b)

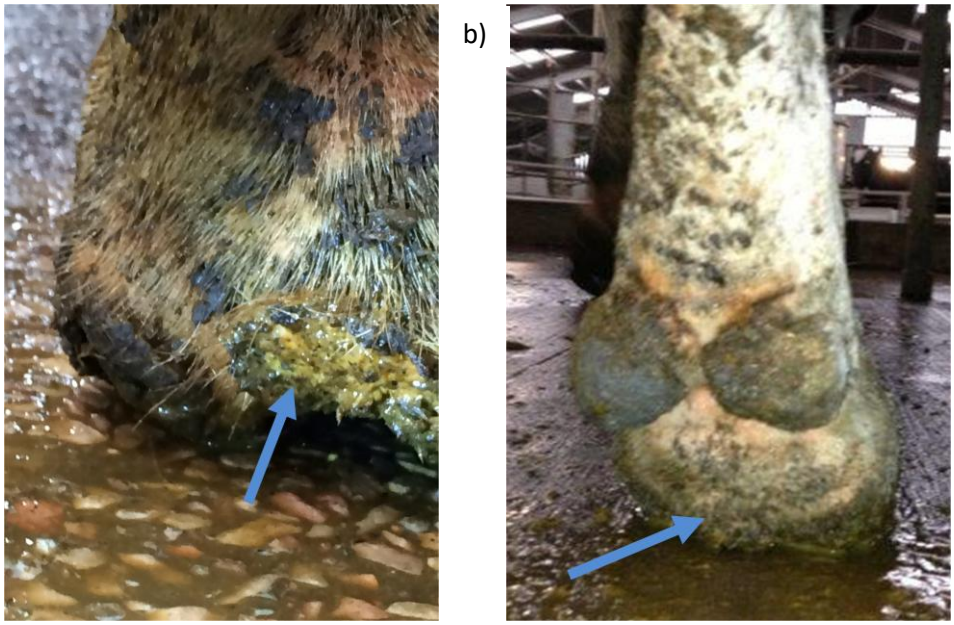


Figure 4.5: DD lesions in the parlour during footprint survey

a) Arrow points to a M2 (active) DD lesion on the plantar aspect of the foot, between the bulbs of the heel, greater than 2cm in diameter and with a moist yellow-grey appearance. b) Arrow points to a M4 lesion on the plantar aspect of the foot, between the bulbs of the heel with proliferative growth in contact with the parlour floor. Photograph credit: Figure a) own photograph and b) gifted by Dr Roger Blowey (Wood Veterinary Group, Gloucestershire, UK).

4.2.4 DNA extraction

Tissue and swab samples (foot trimming equipment and foot prints) were thawed and underwent DNA extraction with the DNeasy Blood and Tissue kit (Qiagen, Manchester, UK) according to the manufacturer's instructions (Appendix A).

Faeces and mucin casts underwent DNA extraction with the Fast DNA Stool Mini kit (Qiagen, Manchester, UK) with the alternative lysis method (QS.1) as described in Chapter 3, Section 3.2.8. Briefly, DNA extraction was carried out according to the manufacturer's instructions with the exception of the lysis step where an alternative lysis incubation temperature of 95°C was employed. Faeces (n=1) and mucin casts (n=14) collected previously by another student underwent DNA extraction using the original protocol of the DNA Stool Mini Kit following the manufacturer's instructions (Qiagen, Manchester, UK).

Feed samples underwent pre-treatment with liquid nitrogen homogenisation. Briefly, samples were immersed in liquid nitrogen for ~12 seconds and homogenised with a pestle and mortar. DNA extraction was then carried out using the PowerSoil® DNA Isolation Kit

(MP; MO BIO laboratories Inc, Carslbad, CA, USA) according to the manufacturer's instructions (Appendix A); however, an extra centrifugation step for 3 minutes was added at step 12 in the manufacturer's instructions and all centrifugation steps from step 12 onwards were carried out at 4˚C.

In order to extract genomic DNA from water collected from water troughs, 1.5 ml of each sample was initially centrifuged at 13 500 rpm for 5 minutes. The supernatant was discarded and the remaining pellet of material underwent DNA extraction with the DNeasy blood and tissue kit (Qiagen, Manchester, UK) according to the manufacturer's instructions (Appendix A).

DNA was extracted from isolated cultures using the chelex resin method (Chua et al., 2005) as described in Chapter 2 Section 2.6.1.

Extracted genomic DNA was aliquoted and stored at -20°C for subsequent PCR analysis.

4.2.5 PCR assays for the detection of DD treponemes

Extracted cultures of isolated spirochaetes were subjected to the universal bacterial 16S rRNA gene PCR assay described in Chapter 2, Section 2.7.1.

Nested PCR assays specific for each of the three DD treponeme phylogroups (*T. medium*, *T. phagedenis* and *T. pedis*) were carried out on extracted genomic DNA from all tissue, trimming equipment swabs and footprint swabs as described in Chapter 2, Section 2.7.2. Faeces and mucin casts collected by the previous student involved with this project also underwent these nested PCRs for the detection of the three DD treponeme phylogroups.

DD treponeme phylogroup *T. medium*, *T. phagedenis* and *T. pedis* specific single step PCR assays previously optimised in Chapter 3, Section 3.2.1 were carried out on extracted genomic DNA from faeces, mucin casts, feed and water samples.

All extracted genomic DNA from samples collected underwent a *Treponema* genus specific 16S rRNA gene targeted PCR assay using primers (Table 2.4) which amplifies DNA belonging to both commensal and pathogenic treponemes as described in Chapter 2, Section 2.7.3.

All PCR assays were carried out in triplicate (as described in Chapter 2 Section 2.7.2). Each PCR assay included genomic DNA from each of the three DD treponeme phylogroups to act as positive or negative controls depending upon the DD treponeme phylogroup the assay

was specific for. Water was used as a negative control for all assays. Results of PCR assays were visualised via agarose gel electrophoresis as described in Chapter 2, Section 2.7.4.

4.2.6 16S rRNA gene sequencing

PCR product purification and sequencing of purified PCR products was carried out as described in Chapter 2, Sections 2.8.1-2.

4.2.7 Phylogenetic tree analysis of 16S rRNA gene sequencing

Phylogenetic analysis of the 16S rRNA gene consensus sequences obtained were carried out in order to determine the relatedness of isolated spirochaetes to pathogenic and commensal treponemes. A TN93 model was used to construct a maximum-likelihood tree with 10 000 bootstrap values (Tamura and Nei, 1993), as described in Chapter 2 Section 2.8.3.

4.2.8 Statistical analysis

Statistical analysis was carried out in collaboration with Dr Joseph Angell an Honorary Fellow at the University of Liverpool, UK and Veterinary Surgeon at Wern Veterinary Surgeons, Ruthin, UK.

Fisher's exact tests were employed on PCR data to investigate:

1) The association between the presence of DD treponemes (*T. medium, T. phagedenis* and *T. pedis* phylogroups and irrespective of phylogroup) in the GI tact and the time of year the tissues were sampled.
2) The association between the presence of DD treponemes (*T. medium, T. phagedenis* and *T. pedis* phylogroups and irrespective of phylogroup) in healthy feet or DD lesions, and the time of year in which the tissues were sampled.
3) The association between the presence of the *Treponema* genus (inclusive of pathogenic and commensal treponemes) in the GI tract or healthy foot tissue and the time of year in which these tissues were sampled.
4) The association between the presence of DD treponemes (*T. medium, T. phagedenis* and *T. pedis* phylogroups and irrespective of phylogroup) on foot trimming equipment and/or gloves with the DD status of the foot being trimmed.
5) The association between the presence of DD treponemes (*T. medium, T. phagedenis* and *T. pedis* phylogroups and irrespective of phylogroup) in cattle footprints and the DD status of the foot leaving the footprint.

6) The association between the presence of DD treponemes (*T. medium, T. phagedenis* and *T. pedis* phylogroups and irrespective of phylogroup) in footprints and the type of floor material (rubber, concrete or metal) they were imparted on.

Fisher's exact test was used to investigate whether there was a statistical association between these nominal variables using contingency tables (Fisher, 1922). Fisher's exact test was chosen as the probability (*P)* value given is more conservative than other tests when the sample size is small, as investigated here.

The critical *P* value for significance for each data set was adjusted using the Bonferroni correction (Dunn, 1961):

$$\alpha' = \alpha/n$$

α' = adjusted critical *P* value

α = critical P value (0.05)

n = number of tests

The Bonferroni correction allows for a more a conservation interpretation of the strength of any association found and therefore reduces the risk of type I error. In addition, Clopper-Pearson 95% confidence intervals (95% CI) (Clopper and Pearson, 1934) were computed for each percentage presented. The 95% CI gives a range about the percentage in which we can be 95% certain the true percentage lies. Both the 95% CIs and the *P* values were used to assess the strength of the associations reported. Whilst the adjusted critical *P* values were used as a basis to determine significance, it was considered more meaningful to take a less prescriptive approach when discussing significance and thus values close to the adjusted critical *P* value were considered to give weak support of an association as described by Angell et al., (2018) and thus were discussion worthy.

Statistical tests were implemented using STATA V.15. (StataCorp, Texas, USA).

4 .3 Results

4.3.1 *Treponema* genus and DD treponeme phylogroup specific PCR survey of various dairy cow tissues

Gingiva, RAJ, DD lesions, healthy foot tissue and 'other' foot tissue collected from dairy cows over a two year period underwent PCR assays specific for the *Treponema* genus and three cultivable DD treponeme phylogroups (*T. medium, T. phagedenis* and *T. pedis*) to

identify potential DD treponeme infection reservoirs. The results of the PCR assays are shown in Tables 4.3-4.7 with results split into those obtained from DD-affected and unaffected cows and into which quarter of the year they were sampled from (January-March, April-June, July-September and October-December).

4.3.1.1 Bovine gastrointestinal tract tissue PCR results

The PCR assays specific for the three cultivable DD treponeme phylogroups (*T. medium, T. phagedenis* and *T. pedis*) detected DD treponemes in 8/122 (6.6%) of gingiva sampled; with each DD treponeme phylogroup (*T. medium¸ T. phagedenis* and *T. pedis*) present in 3/122 (2.5%) samples (Table 4.3). Only one gingiva sample (0.8%) was positive for more than one of the investigated DD treponeme phylogroups. Gingiva samples were collected from ten DD-affected cows and 112 DD-unaffected cows. Of the *T. phagedenis* positive samples, one was obtained from a DD-affected cow whereas the other two *T. phagedenis* positive samples were obtained from DD-unaffected cows. *T. medium* and *T. pedis* DD treponeme phylogroups were only detected in samples from DD-unaffected cows. DD treponeme positive gingival tissues were obtained from samples collected in both the housing season (Oct-Mar) and grazing season (Apr-Sept). With regards to the *Treponema* genus PCR assays (which includes both pathogenic and commensal treponemes), 63/122 (51.6%) gingiva samples were positive for the *Treponema* genus with 5/10 (50%) and 58/112 (51.8%) gingiva positive for the treponemal DNA in DD-affected and DD-unaffected animals, respectively.

DD treponemes were detected in only 1/121 (0.8%) RAJ samples subjected to the *T. medium, T. phagedenis* and *T. pedis* DD treponeme phylogroup specific PCR assays (Table 4.4). The RAJ sample was positive for the *T. phagedenis* DD treponeme phylogroup and was obtained from one of the ten DD-affected cows (both feet affected) during the grazing season. DNA from the *Treponema* genus was detected in 80/121 (66.1%) of RAJ samples with 5/10 (50%) DD-affected cows positive and 75/111 (67.6%) DD-unaffected cows positive for the *Treponema* genus.

4.3.1.2 Bovine pedal tissue sample results

Samples obtained from the region of the hind feet where the horn meets the skin, between the bulbs of the heel, were classified into three different groups: DD lesions, healthy feet and ‘other’ feet samples. The ‘other’ feet sample group was created due to the ambiguity

of health observed in some feet, where the skin abnormalities present could not be described as DD lesions but also could not be described as healthy (See Section 4.2.2).

Table 4.3: PCR and isolation results for gingiva samples from dairy cows surveyed for the presence of DD treponemes

			Culture of spirochaetes		PCR assay detection [e]			
Season[a]	**DD status[b]**	**No. of samples**	**Observed[c]**	**Isolation [d]**	**1**	**2**	**3**	**T**
Jan-Mar	+	1	-	-	-	-	-	1
Jan-Mar	-	30	5	60B (2 NIA)	1	1	-	17
Apr-Jun	+	6	-	- (2 NIA)	-	1	-	2
Apr-Jun	-	24	-	- (2 NIA)	2	-	1	11
Jul-Sept	+	2	-	-	-	-	-	1
Jul-Sept	-	28	4	-	-	1	-	12
Oct-Dec	+	1	-	-	-	-	-	1
Oct-Dec	-	30	3	- (3 NIA)	-	-	2	18
Total		122	12	1	3	3	3	63

[a] Samples were split into the quarter of the year in which they were obtained. Oct-Dec and Jan-Mar generally compose the housing season and Apr-Jun and July-Sept compose the grazing season.

[b] (+) refers to DD-affected cows with a DD lesion on at least one hind foot typical of DD and (-) denotes DD-unaffected cows with no visible DD lesions (category includes cows with 'other' foot problems).

[c] Number of cultures where spirochaete-like morphology was observed by phase contrast microscopy. (-) denotes no spirochaete-like morphology observed.

[d] Isolate names listed, NIA denotes no isolation attempted. (-) denotes all isolations failed.

[e] 1, 2, 3 and T denote the T. medium, T. phagedenis, T. pedis DD treponeme phylogroup specific nested PCR assays respectively and the *Treponema* genus specific PCR assay respectively. *The frequency of positive PCR results for each assay is shown where (-) denotes no positive results.*

Of the twelve DD lesion samples collected during this study, 100% were PCR positive for one or more of the *T. medium, T. phagedenis* and *T. pedis* DD treponeme phylogroups (Table 4.5). With regards to the DD treponeme phylogroups individually, 7/12 (58.3%), 12/12 (100%) and 5/12 (41.7%) were positive for DNA from the *T. medium*, *T. phagedenis* and *T. pedis* DD treponeme phylogroups respectively. The PCR assays showed that 8/12 (66.6%) of DD lesions were positive for more than one of the *T. medium, T. phagedenis* and *T. pedis* DD treponeme phylogroups with a third positive for two phylogroups and a third positive for all three phylogroups investigated.

Table 4.4: PCR and isolation results for RAJ samples from dairy cows surveyed for the presence of DD treponemes

			Culture of spirochaetes		PCR assay detection [e]			
Season[a]	**DD status[b]**	**No. of samples**	**Observed[c]**	**Isolation[d]**	**1**	**2**	**3**	**T**
Jan-Mar	+	1	-	-	-	-	-	-
Jan-Mar	-	30	4	- (2 NIA)	-	-	-	20
Apr-Jun	+	6	-	-	-	-	-	3
Apr-Jun	-	23	-	- (1 NIA)	-	-	-	17
Jul-Sept	+	2	-	-	-	1	-	1
Jul-Sept	-	28	3	-	-	-	-	15
Oct-Dec	+	1	-	-	-	-	-	1
Oct-Dec	-	30	1	- (3 NIA)	-	-	-	23
Total		121	8	1	0	1	0	80

[a] *Samples were split into the quarter of the year in which they were obtained. Oct-Dec and Jan-Mar generally compose the housing season and Apr-Jun and July-Sept compose the grazing season.*

[b] *(+) denotes DD-affected cows with a DD lesion on at least one hind foot typical of DD and (-) denotes DD unaffected cows with no visible DD lesions (category includes cows with 'other' foot problems).*

[c] *Number of cultures where spirochaete-like morphology was observed by phase contrast microscopy. (-) denotes no spirochaete-like morphology observed.*

[d] *NIA denotes no isolation attempted. (-) denotes all isolations failed.*

[e] *1, 2, 3 and T denote the T. medium, T. phagedenis, T. pedis DD treponeme phylogroup specific nested PCR assays respectively and the Treponema genus specific PCR assay respectively. The frequency of positive PCR results for each assay is shown where (-) denotes no positive results.*

DD lesion PCR data collected during this study was compared with PCR results from 29 DD lesions sampled between the years 2003 and 2007 by Evans et al., (2009c) (Table 4.5). The Evans et al., (2009c) study used the same extraction and PCR assay methods as described in this thesis although sample collection differed in that a 3 mm punch biopsy of the DD lesions was taken from cattle on farms. Similar to the results of this thesis, 100% of DD lesions collected between 2003 and 2007 were positive for the *T. phagedenis* DD treponeme phylogroup. Also in concurrence with this thesis, the *T. medium* DD treponeme phylogroup was the second most prominent phylogroup with 28/29 (96.6%) DD lesions positive followed by the *T. pedis* DD treponeme phylogroup with 21/29 (72.4%) DD lesions positive. However, a larger percentage of DD lesions were positive for the *T. medium* and *T. pedis* DD treponeme phylogroups in the Evans et al., (2009c) study than was found in lesions examined in this thesis which were 7/12 (58.3%) and 5/12 (41.7%) respectively . All three DD treponeme phylogroups (*T. medium, T. phagedenis* and *T.* pedis) were present in almost three quarters (72.4%) of lesions investigated in the Evans et al., (2009c) study whereas only a third (33.3%) of the DD lesions in this study were positive for all three

phylogroups. Additionally in this thesis, the *T. pedis* DD treponeme phylogroup was not detected in DD lesions collected in January-March and October-December when cattle may be housed, however, in the Evans et al., (2009c) study the *T. pedis* DD treponeme phylogroup could be detected across all quarters of the year. This difference is likely due to the small number of samples obtained in this study compared to the Evans et al., (2009c) study and thus caution must be taken when comparing these two data sets.

Table 4.5: PCR and isolation results for DD lesion samples from dairy cows surveyed for the presence of DD treponemes during this thesis and compared with PCR results from lesions collected between 2003-2007 in parentheses (Evans et al., 2009c).

Season[a]	No. of samples	Culture of spirochaetes		PCR assay detection[d]			
		Observed[b]	Isolation[c]	1	2	3	T
Jan-Mar	2 [9]	1	- (1 NIA)	1 [9]	2 [9]	- [9]	2 [9]
Apr-Jun	6 [8]	4	-	3 [8]	6 [8]	3 [6]	6 [8]
Jul-Sept	3 [4]	-	-	2 [4]	3 [4]	2 [3]	3 [4]
Oct-Dec	1 [8]	-	-	1 [7]	1 [8]	- [3]	1 [8]
Total	12 [29]	5	0	7 [28]	12[29]	5 [21]	12 [29]

[a] *Samples were split into the quarter of the year in which they were obtained. Oct-Dec and Jan-Mar generally compose the housing season and Apr-Jun and July-Sept compose the grazing season.*

[b] *Number of cultures where spirochaete-like morphology was observed by phase contrast microscopy. (-) denotes no spirochaete-like morphology observed.*

[c] *NIA denotes no isolation attempted. (-) denotes all isolations failed.*

[d] *1, 2, 3 and T denote the T. medium, T. phagedenis, T. pedis DD treponeme phylogroup specific nested PCR assays respectively and the Treponema genus specific PCR assay respectively. The frequency of positive PCR results for each assay is shown where (-) denotes no positive results.*

When subjected to the three DD treponeme phylogroup (*T. medium*, *T. phagedenis* and *T. pedis*) specific nested PCR assays, 2/16 (12.5%) 'other' feet samples were positive for one or more of the DD treponeme phylogroups (Table 4.6). Indeed, one foot sample was positive for all three DD treponeme phylogroups whereas the other positive foot sample was only positive for the *T. phagedenis* DD treponeme phylogroup. All 'other' foot samples were obtained from DD-unaffected cows. Of the positive 'other' foot samples, one was sampled in the latter half of the grazing season (July-September) whereas the other was sampled in the early half of the housing season (October-December). With regards to the *Treponema* genus specific PCR assay, 9/16 (56.3%) of 'other' foot samples were positive.

Table 4.6: PCR and isolation results for 'other' foot samples surveyed for the presence of DD treponemes

			Culture of spirochaetes		PCR assay detection [e]			
Season[a]	**DD status[b]**	**No. of samples**	**Observed[c]**	**Isolation[d]**	**1**	**2**	**3**	**T**
Jan-Mar	-	2	1	-	-	-	-	2
Apr-Jun	-	6	2	-	-	-	-	1
Jul-Sept	-	4	1	-	-	1	-	2
Oct-Dec	-	4	2	-	1	1	1	4
Total		16	6	0	1	2	1	9

[a] Samples were split into the quarter of the year in which they were obtained. Oct-Dec and Jan-Mar generally compose the housing season and Apr-Jun and July-Sept compose the grazing season.

[b] (-) denotes DD unaffected cows with no visible DD lesions.

[c] Number of cultures where spirochaete-like morphology was observed by phase contrast microscopy. (-) denotes no spirochaete-like morphology observed.

[d] (-) denotes all isolations failed.

[e] 1, 2, 3 and T denote the T. medium, T. phagedenis, T. pedis DD treponeme phylogroup specific nested PCR assays respectively and the Treponema genus specific PCR assay respectively. The frequency of positive PCR results for each assay is shown where (-) denotes no positive results.

Unusually, DD treponemes were detected in 39/217 (18%) healthy foot samples subjected to the PCR assays for the *T. medium, T. phagedenis* and *T. pedis* DD treponeme phylogroups (Table 4.7). When examining the individual DD treponeme phylogroups, 20/217 (9.2%), 35/217 (16.1%) and 13/217 (6%) healthy feet were positive for the *T. medium, T. phagedenis* and *T. pedis* DD treponeme phylogroups, respectively. Furthermore, 11/217 (5%) of the healthy feet were positive for two of the DD treponeme phylogroups and 9/217 (4.1%) were positive for all three DD treponeme phylogroups investigated. Healthy feet were collected from nine DD-affected animals, where the other foot had a DD lesion present, and 208 healthy feet were collected from 111 DD-unaffected cows (including cows where a foot was classified as 'other'). Of the DD treponeme positive healthy feet samples, five were obtained from DD-affected animals and the other 34 DD treponeme positive samples were from DD-unaffected animals.

When analysing the findings in terms of spread across the year, DD treponemes were detected in healthy feet obtained in all four quarters of the year (January-March, April-June, July-September and October to November). The largest proportion of healthy feet positive for the *T. medium* DD treponeme phylogroup were obtained between January and March (latter half of housing season) with 9/217 (4.1%) of the total healthy feet positive. *T. phagedenis* DD treponeme phylogroup detection rates were also highest in January-March (13/217, 6%) followed by a small reduction in detection in April-June (10/217, 4.6%) and

July-September (8/217, 3.7%). The lowest detection rate of *T. phagedenis* DD treponeme phylogroup detection was in the months spanning October-December (4/217, 1.8%). The *T. pedis* DD treponeme phylogroup generally had a lower detection rate than the other two DD treponeme phylogroups and unlike the other two, the *T. pedis* DD treponeme phylogroup experienced its highest rates of detection during April-June (6/217, 2.8%). The *Treponema* genus specific PCR assay showed that 122/217 (56.2%) healthy foot samples were positive for both commensal and pathogenic treponemes, with no preference for DD-affected or DD-unaffected cows.

Table 4.7: PCR and isolation results for healthy foot samples from dairy cows surveyed for the presence of DD treponemes

			Culture of spirochaetes		PCR assay detection [e]			
Season[a]	**DD status[b]**	**No. of samples**	**Observed[c]**	**Isolation [d]**	**1**	**2**	**3**	**T**
Jan-Mar	+	1	-	-	-	1	-	1
Jan-Mar	-	58	9	59A (6 NIA)	9	12	3	35
Apr-Jun	+	6	3	-	1	3	1	4
Apr-Jun	-	42	9	226A , 253A, (2 NIA)	5	7	5	23
Jul-Sept	+	1	-	-	-	-	-	-
Jul-Sept	-	52	5	-	3	8	3	27
Oct-Dec	+	1	-	-	1	1	-	1
Oct-Dec	-	56	4	- (5 NIA)	1	3	1	31
Total		217	30	3	20	35	13	122

[a] *Samples were split into the quarter of the year in which they were obtained. Oct-Dec and Jan-Mar generally compose the housing season and Apr-Jun and July-Sept compose the grazing season.*

[b] *(+) denotes DD-affected cows with a DD lesion on at least one hind foot typical of DD and (-) denotes DD unaffected cows with no visible DD lesions (category includes cows with 'other' foot problems).*

[c] *Number of cultures where spirochaete-like morphology was observed by phase contrast microscopy. (-) denotes no spirochaete-like morphology observed.*

[d] *Isolate names listed. NIA denotes no isolation attempted. (-) denotes all isolations failed.*

[e] *1, 2, 3 and T denote the T. medium, T. phagedenis, T. pedis DD treponeme phylogroup specific nested PCR assays respectively and the Treponema genus specific PCR assay respectively. The frequency of positive PCR results for each assay is shown where (-) denotes no positive results.*

4.3.2 *Treponema* genus and DD treponeme phylogroup specific PCR survey of dairy farm environment samples linked to the gastrointestinal tract

Faeces, mucin casts, feed and water samples underwent PCR assays specific for the three cultivable phylogroups of DD treponemes (*T. medium, T. phagedenis* and *T. pedis*) as well as the *Treponema* genus. The results of the faeces and mucin cast PCR assays are shown in Tables 4.8.

A total of 62 faecal samples were collected from two dairy farms (A and B, Table 4.1) where DD was endemic. Collection of faeces spanned the months of February, April, June, November and December. Mucin casts were also collected from farm A (Table 4.1) (*n*=31) by sieving faecal samples with water to obtain mucin casts. Mucin casts were collected in the months of February, March, June, November and December. All faecal and mucin cast samples were negative by PCR for the *T. medium, T. phagedenis* and *T. pedis* DD treponeme phylogroups (Table 4.8). On the other hand, the *Treponema* genus PCR assay detected *Treponema* DNA in 61/62 (98.2%) and 29/31 (93.5%) faecal and mucin cast samples respectively.

Samples of mixed ration feed (*n*=20) were collected from various housing areas (low yield, high yield etc) in Farm A which is DD endemic. Of these feed samples four were halved and allowed to spoil for 3 months before analysis. The University of Liverpool Veterinary School (Liverpool, UK) also provided 16 samples of individual components of feed for cattle and other animals. Water samples (*n*=19) from water troughs were collected from various housing areas (low yield, high yield etc) in two dairy farms (A and F, Table 4.1). All feed and water samples were negative for DNA from the *T. medium¸ T. phagedenis* and *T. pedis* DD treponeme phylogroups as well as the *Treponema* genus, following the respective PCR assays.

4.3.3 *Treponema* genus and DD treponeme phylogroup specific PCR survey of hoof trimming equipment

Foot trimming equipment (including hoof grinders and clippers) as well as gloves before and after use underwent PCR assays for the detection of the three cultivable DD treponeme phylogroups (*T. medium, T. phagedenis* and *T. pedis*) and the *Treponema* genus in general. The overall results of these PCR assays are shown in Table 4.9 (gloves) and 4.10 (trimming equipment).

Table 4.8: PCR and isolation results for dairy bovine faeces and mucin cast samples surveyed for the presence of DD treponemes

Source	DD status[a]	Number of samples	Culture of spirochaetes		PCR assay detection[d]			
			Observed[b]	Isolation[c]	1	2	3	T
faeces	+	42	7	F264 (25 NIA)	-	-	-	41
faeces	-	15	5	F803 (3 NIA)	-	-	-	15
faeces	/	5	1	- (3 NIA)	-	-	-	5
mucin casts	+	7	NT	- (7 NIA)	-	-	-	5
mucin casts	-	2	NT	- (2 NIA)	-	-	-	2
mucin casts	/	22	6	HY5, HY7 (6 NIA)	-	-	-	22
Total		93	19	4	0	0	0	90

[a] *(+) denotes DD-affected cows with a DD lesion on at least one hind foot typical of DD and (-) denotes DD unaffected cows with no visible DD lesions (category includes cows with 'other' foot problems).*

[b] *Number of cultures where spirochaete-like morphology was observed by phase contrast microscopy. (-) denotes no spirochaete-like morphology observed.*

[c] *Isolate names listed. NIA denotes no isolation attempted. (-) denotes all isolations failed.*

[d] *1, 2, 3 and T denote the T. medium, T. phagedenis, T. pedis DD treponeme phylogroup specific nested PCR assays respectively and the Treponema genus specific PCR assay respectively. The frequency of positive PCR results for each assay is shown where (-) denotes no positive results.*

During visitation by the foot trimmer, gloves were replaced between each cow and swabs taken prior to foot trimming as well as after trimming (Table 4.9). Gloves swabbed before foot trimming were negative by PCR for DD treponemes belonging to the *T. medium, T. phagedenis and* T. pedis phylogroups. However, after foot trimming DD treponemes were detected on 9/16 (56.3%) gloves. Positive PCR results were only obtained after trimming DD-affected feet; all gloves used on DD-unaffected cows were negative for DD treponemes after foot trimming. Furthermore in terms of phylogroups, DD treponemes belonging to the *T. medium and T. phagedenis phylogroup* were detected on 4/10 (40%) and 9/10 (90%) gloves respectively. Only 2/11 gloves prior to use were positive for the *Treponema* genus, on the other hand, 100% of gloves after trimming were positive for the *Treponema* genus irrespective of DD status of the foot trimmed.

Table 4.9 PCR results of gloves prior to and following use on each cow during foot trimming surveyed for the presence of DD treponemes

Gloves[a]	DD status[b]	Number of samples	PCR assay detection [c]			
			1	2	3	T
Prior to use	+	3	-	-	-	-
Prior to use	-	8	-	-	-	2
After trim	+	10	4	9	-	10
After trim	-	6	-	-	-	6
Total		27	4	9	-	18

[a] *A fresh pair of gloves were put on for each cow.*

[b] *(+) denotes DD-affected cows with a DD lesion on at least one hind foot typical of DD and (-) denotes DD unaffected cows with no visible DD*

[c] *1, 2, 3 and T denote the T. medium, T. phagedenis, T. pedis DD treponeme phylogroup specific nested PCR assays respectively and the Treponema genus specific PCR assay respectively. The frequency of positive PCR results for each assay is shown where (-) denotes no positive results.*

The hoof grinder disc and handle were swabbed following use on cattle feet during foot trimming. DD treponeme DNA was detected on 1/24 (4.2%) hoof grinder discs swabbed, which was positive for both the *T. medium* and *T. phagedenis* DD treponeme phylogroups. The *T. pedis* DD treponeme phylogroup was not detected on any hoof grinder discs. The positive hoof grinder disc had been swabbed following trimming of a DD-unaffected foot. DD treponeme DNA belonging to the *T. phagedenis* DD treponeme phylogroup was also detected on 1/19 (5.3%) swabs of the hoof grinder handle swabbed following use on a DD-unaffected foot (different cow to the positive hoof grinder disc). DNA from the *Treponema* genus was detected on 10/24 (41.7%) hoof grinder discs. The hoof grinder handle, however, had a higher detection rate for the *Treponema* genus with 18/19 (94.7%) positive.

Clippers were swabbed following foot trimming of each foot per cow, washed briefly in cold water and swabbed again. Following foot trimming 2/20 (10%) clippers were positive for DD treponemes belonging to the *T. phagedenis* DD treponeme phylogroup. All clippers were negative for the *T. medium* and *T. pedis* DD treponeme phylogroups. Clippers positive for DD treponemes were obtained following the foot trimming of DD-affected feet. Following washing in cold water DD treponemes could no longer be detected by PCR on the clippers in which they were detected before washing (n=2). Furthermore, clippers were 100% (20/20) positive for *Treponema* genus DNA following foot trimming, however, the 18 clippers that were swabbed again following washing in water became negative for the *Treponema* genus.

Overall when foot trimming tool (clippers, hoof grinder disc and hoof grinder handle) results were combined 4/63 (6.3%) were positive for DD treponemes (Table 4.10). The *T. medium* and *T. phagedenis* DD treponeme phylogroups were detected on 1/63 (1.6%) and 4/63 (6.3%) of the foot trimming tools sampled respectively. Of the foot trimming tools used on DD-affected feet, 2/11 were positive for DD treponemes; specifically the *T. phagedenis* DD treponeme phylogroup. On the other hand 1/52 and 2/52 of foot trimming tools used on DD-unaffected feet were positive for the *T. medium* and *T. phagedenis* DD treponeme phylogroups respectively. DNA belonging to the *Treponema* genus (pathogenic and commensal treponemes) was detected on foot trimming tools used on 9/11 (81.8%) and 39/52 (75%) DD-affected and DD-unaffected feet respectively.

Table 4.10: PCR results for trimming tools (clippers and hoof grinder disc and handle) surveyed for the presence of DD treponemes

		PCR assay detection [b]			
DD status[a]	**No. of samples**	**1**	**2**	**3**	**T**
+	11	-	2	-	9
-	52	1	2	-	39
Total	63	1	4	0	48

[a] (+) denotes DD-affected cows with a DD lesion on at least one hind foot typical of DD and (-) denotes DD unaffected cows with no visible DD

[b] 1, 2, 3 and T denote the T. medium, T. phagedenis, T. pedis DD treponeme phylogroup specific nested PCR assays respectively and the Treponema genus specific PCR assay respectively. The frequency of positive PCR results for each assay is shown where (-) denotes no positive results.

4.3.4 *Treponema* genus and DD treponeme phylogroup specific PCR survey of foot prints on dairy farm floor surfaces

Footprints created by cow feet on the floor where cows may stand for a period of time were surveyed to ascertain whether DD treponemes were present. Furthermore, footprints on three different types of floor material: rubber, metal and concrete typically used in dairy farms settings were compared for the presence of DD treponemes by PCR. Results of the *T. medium, T. phagedenis* and *T. pedis* specific nested PCR assays as well as the *Treponema* genus PCR assay are shown in Table 4.11.

A total of 169 footprints were surveyed across four farms for the presence of DD treponemes. Overall 22/169 (13%) footprints were positive by PCR for DD treponemes. Footprints from DD-affected feet accounted for 18 of the DD treponeme PCR positive

results. The remaining 4 positive results were from DD-unaffected feet. With regards to the individual DD treponeme phylogroups, 13/169 (7.7%), 19/169 (11.2%) and 5/169 (3%) were positive for DNA from the *T. medium, T. phagedenis* and *T. pedis* DD treponeme phylogroups respectively. For DD-affected feet, *T. medium, T. phagedenis* and *T. pedis* DD treponeme phylogroups were present in 11/89 (12.4%), 16/89 (18%) and 4/89 (4.5%) respectively. On the other hand, footprints from DD-unaffected feet showed a marked reduction in detection of the individual DD treponeme phylogroups with 2/80 (2.5%), 3/80 (3.8%) and 1/80 (1.3%) positive for DNA from the *T. medium, T. phagedenis* and *T. pedis* DD treponeme phylogroups. A proportion (10/169, 5.9%) of footprints were positive for more than one DD treponeme phylogroup with 5 footprints positive for all three cultivable DD treponeme phylogroups.

DD treponemes were detected in 11/72 (15.3%), 10/53 (18.9%), 1/44 (2.3%) footprints surveyed on rubber, concrete and metal floors respectively (Table 4.11). The *T. medium* DD treponeme phylogroup was detected in 7/72 (9.7%) and 6/53 (11.3%) footprints on rubber and concrete floors respectively. Additionally, the *T. pedis* DD treponeme phylogroup was detected in 2/72 (2.8%) and 3/53 (5.7%) footprints on rubber and concrete floors respectively. However, the *T. medium* and *T. pedis* DD treponeme phylogroups were not detected in footprints on metal floors. In contrast the *T. phagedenis* DD treponeme phylogroup was detected in footprints on all floor types with 9/72 (12.5%), 9/53 (17%), 1/44 (2.3%) footprints positive on rubber, concrete and metal floors respectively.

For all three floor material types, a larger proportion of footprints were positive for DD treponemes when the footprint was made by a DD-affected foot (Table 4.11). Of DD-affected footprints on rubber floors, 6/31 (19.4%), 7/31 (22.6%) and 2/31 (6.5%) were positive for *T. medium, T. phagedenis and T. pedis* DD treponemes phylogroups respectively whereas 1/41 (2.4%), 2/41 (4.9%), 0/41 (0%) of DD-unaffected footprints on rubber floors were positive for *T. medium*, *T. phagedenis* and *T. pedis* DD treponeme phylogroups by PCR. For DD-affected footprints on concrete floors, 5/38 (13.2%), 8/38 (21.1%) and 2/38 (5.2%) were PCR positive for the *T. medium, T. phagedenis* and *T. pedis* DD treponeme phylogroups whereas 1/38 (2.6%) for each of the three DD treponeme phylogroups was positive for DD-unaffected footprints on concrete floors. For metal floor footprints, the only positive footprint (*T. phagedenis* DD treponeme phylogroup) was from a DD-affected foot (1/20, 5%).

The *Treponema* genus PCR showed that 164/169 (97.1%) footprints were positive for treponemes in general (both commensal and pathogenic) (Table 4.11). Treponemal DNA was detected on 100% of footprints on concrete and metal floors. However, a few negative results were obtained from footprints on rubber floors for the *Treponema* genus with 67/72 (93.1%) positive by PCR.

Table 4.11: PCR results for footprints on different floor surfaces surveyed for the presence of DD treponemes

Floor surface[a]	DD status[b]	No. of samples	PCR assay detection [c]			
			1	2	3	T
Rubber	+	31	6	7	2	30
Rubber	-	41	1	2	-	37
concrete	+	38	5	8	2	38
concrete	-	15	1	1	1	15
metal	+	20	-	1	-	20
metal	-	24	-	-	-	24
Total		169	13	19	5	164

[a] *Footprints were swabbed on three types of floor surface*.

[b] *(+) denotes DD-affected cows with a DD lesion on at least one hind foot typical of DD and (-) denotes DD unaffected cows with no visible DD*

[c] *1, 2, 3 and T denote the T. medium, T. phagedenis, T. pedis DD treponeme phylogroup specific nested PCR assays respectively and the Treponema genus specific PCR assay respectively. The frequency of positive PCR results for each assay is shown where (-) denotes no positive results.*

Where possible, floors were washed with water or in the case of concrete floors a very dilute peracetic acid, and slurry removed between each cow. A small subset of swabs were taken of the floor where the hind feet would likely stand following washing. All washed floors were negative for DD treponemes. However, washing did not completely remove treponemal DNA with 18/26 (69.2%) positive with the *Treponema* genus PCR.

4.3.5 Statistical significance of DD treponeme PCR surveys

Statistical analyses of the infection reservoir PCR survey results were carried out using the Fisher's Exact test. Statistical significance was set at $P < 0.05$, adjusted for repeated measures using the Bonferroni correction. These P values and 95% CIs were used to assess the strength of the associations investigated.

There was no association between the presence of either the *T. medium, T. phagedenis* or *T. pedis* DD treponeme phylogroups, DD treponemes irrespective of phylogroup or the

Treponema genus in the GI tract and the housing (October-March) and grazing (April-September) seasons (critical *P value* for significance is 0.01).

There was also no association (*P* < critical *P* value of 0.01) between the presence of either the *T. medium, T. phagedenis* or *T. pedis* DD treponeme phylogroups, DD treponemes irrespective of phylogroup or the *Treponema* genus in healthy foot tissues and the aforementioned housing and grazing seasons (Table 4.12). However, there was weak evidence of an association (*P* = 0.023, critical *P* value is 0.01) between the presence of DD treponemes (irrespective of phylogroup) in healthy foot tissue and the quarter of the year the tissues were sampled (Table 4.12) but there is overlap of the 95% CIs so although there may be a possible trend further investigation would be necessary to explore this further.

There were no associations (*P* < critical *P* value of 0.013) between the presence of either the *T. medium, T. phagedenis* or *T. pedis* DD treponeme phylogroups or DD treponemes irrespective of phylogroup presence in DD lesions and the housing or grazing seasons as well as the quarter of the year in which they were sampled.

The PCR data from the collated foot trimming tool samples was further combined with the gloves PCR data (and henceforth referred to as 'all foot trimming equipment') to determine whether there was an association between DD status of the foot trimmed and the presence of DD treponemes (*T. medium, T. phagedenis* or *T. pedis* phylogroups or irrespective of phylogroup) on all foot trimming equipment following trimming (Table 4.13). Statistical analysis showed that there was a strong association (*P* = <0.001, critical *P* value is 0.013, no 95% CI overlap) between the presence of DD treponemes irrespective of phylogroup on all foot trimming equipment after trimming and trimming of DD-affected feet. In addition, there was a strong association (*P* = < 0.001, no 95% CI overlap) between the presence of the *T. phagedenis* DD treponeme phylogroup and the trimming of DD-affected feet. There did appear to be weak evidence of an association (*P* = 0.016, critical *P* value is 0.013) between the *T. medium* DD treponeme phylogroup and the trimming of affected feet, however there is some overlap between the 95% CIs suggesting further investigation would be necessary to explore this further.

If the data was divided into trimming tools and gloves after trim, there was no association (*P* < critical *P* value 0.013) between the trimming of DD-affected feet and the presence of DD treponemes (*T. medium, T. phagedenis* and *T. pedis* phylogroups or irrespective of phylogroups) on the trimming tools (Table 4.13). However, there was a strong association

(*P* = 0.001) between the presence of DD treponemes on gloves and the trimming of DD-affected feet; with a strong association (*P* = 0.001) of *T. phagedenis* DD treponeme phylogroup presence on gloves that have just been used to trim DD-affected feet.

Out of interest, the results of the swabs taken of foot trimming tools following trimming were combined with the results from Sullivan et al., (2014a) study for hoof knife blades swabbed following trimming of dairy and beef cattle (Table 4.13). Sullivan et al., (2014a) used the same DNA extraction and PCR methodology as described for swabs collected in this thesis. There were strong associations between the presence of each of the three DD treponeme phylogroups (*P* < 0.001 for *T. medium* and *T. phagedenis phylogroups and P* = 0.001 for the *T. pedis* phylogroup, critical *P* value is 0.013) as well DD treponemes (*P* < 0.001) irrespective of phylogroup on trimming tools (including foot knife blades) following trimming of DD-affected feet.

Statistical analysis was carried out to determine if there was an association between the presence of DD treponemes (*T. medium, T. phagedenis* and *T. pedis* phylogroups or irrespective of phylogroup) in footprints and DD-affected feet (Table 4.14). This analysis demonstrated that there was a strong association (*P* = 0.005, critical *P* value was 0.013) with the presence of DD treponemes in footprints and DD-affected feet. When examining the DD treponeme phylogroups there was a strong association (*P* = 0.003) between the presence of the *T. phagedenis* DD treponeme phylogroup and footprints made by DD-affected feet. There was also weak evidence of an association (*P* = 0.020, critical *P* value is 0.013) between the presence of the *T. medium* DD treponeme phylogroup in footprints and DD-affected feet, however, the overlap of 95% CIs suggesting further investigation would be necessary to explore this further.

Whether there was an association between the presence of DD treponemes in footprints and floor type footprints were sampled from was investigated (Table 4.14). There was no association (*P* > critical *P* value of 0.013) between the presence of either the *T. medium, T. phagedenis* or *T. pedis* DD treponeme phylogroups in footprints and floor type (rubber, concrete and metal). Whilst for the *T. medium and T. pedis* DD treponeme phylogroups there was overlap of the 95% CIs for the concrete and rubber floors, there was no detection on the metal floor types which may be indicative of an association with concrete and rubber compared to metal, but with the small numbers of samples investigated it could only be postulated at this stage. There was weak evidence of an association (*P* = 0.02, critical *P* value is 0.013) between the presence of DD treponemes (irrespective of

phylogroup) in footprints and floor type, however, as previously discussed the overlap of the 95% CI values suggests further investigation would be necessary to explore this further

4.3.6 Culture and isolation of spirochaetes

4.3.6.1 Bovine gastrointestinal tissues

A proportion of gingiva, RAJ, healthy feet, 'other' feet and DD lesional tissue were cultured in culture medium optimised for treponemal growth in order to grow and isolate spirochaetes, particularly treponemes, present in the tissues. Results are shown in Tables 4.3-4.7 as well as Figure 4.6.

Of the 113 gingiva samples cultured, spirochaete-like morphology was observed by phase contrast microscopy in cultures from 12 (10.6%) samples. However, isolation was successful from only one (0.9%) gingiva sample, which was negative by PCR for DD treponemes but positive for the *Treponema* genus. The isolate, 60B, shares over 99% 16S rRNA gene sequence identity with bacterium DAZ1007 (Genbank accession: KF697910.1) originally obtained from a bovine rumen in New Zealand and clusters with the commensal treponemes upon phylogenetic analysis (Figure 4.6). Furthermore, the isolate appears to be phylogenetically closely related to *Treponema bryantii* (Genbank accession: JX218818.1), a commensal treponeme of the rumen. All other isolation attempts from gingiva tissue failed. Spirochaete-like morphology was observed in 8/115 (7%) cultures of inoculated RAJ. However, all isolation attempts failed.

Surprisingly, all isolations from DD lesions failed although spirochaete-like morphology was observed in cultures from 5/11 (45.5%) DD lesion samples (Table 4.5). Similarly all isolations failed from 'other' feet samples but spirochaete-like morphology was observed in 6/16 (37.5%) 'other' feet cultured. On the other hand, spirochaete-like morphology was observed in 30/204 (14.7%) healthy feet samples cultured and three (1.5%) spirochaetes were isolated. Following construction of a maximum likelihood phylogenetic tree (Figure 4.6), two of the spirochaete isolates (named 59A and 253A) clustered with the *T. phagedenis* DD treponeme phylogroup. Indeed, analysis of the 16S rRNA gene sequences showed that these two isolates share 100% sequence identity with *T. phagedenis* strain DD1F (Genbank accession: KR025845.1), originally isolated from a dairy cow DD lesion in the UK (Clegg et al., 2016b). Another isolate obtained from a healthy foot sample, named 226A, shared 94% 16S rRNA gene sequence identity with uncultured bacterium clone HFMBR 2-10 (Genbank accession: JX628614.1) and clustered with the commensal

treponemes upon phylogenetic analysis (Figure 4.6). Additionally, two isolates which share 100% 16S rRNA gene sequence identity to Fusobacterium sp. strain X-13 (Genbank accession: MF188195.1) were also cultured from two healthy feet samples, however, they were not included in further analysis as they were not spirochaetes. Of the samples in which a spirochaete isolate was successfully obtained, two were positive by PCR for DD treponemes.

4.3.6.1 Dairy farm environment samples linked to the bovine gastrointestinal tract

Faecal, mucin cast and feed samples were inoculated into culture medium enriched for treponemal growth with the aim of enabling growth and isolation of spirochaetes from environment samples in order to identify DD treponemes. Results are shown in tables 4.8 and 4.9 as well as Figure 4.6.

Spirochaete-like morphology was observed via phase contrast microscopy in 13/31 (41.9%) and 6/16 (37.5%) of faecal and mucin cast samples cultured respectively. Isolation of spirochaetes was successful from two faecal samples (6.5%) that were positive for the *Treponema* genus PCR. Of those isolates, one (named F264) shared 100% 16S rRNA sequence identity with Ru2 (Genbank accession: GU566701) a commensal treponeme of the ruminant GI tract. The other isolate, named F803, shared 99% sequence identity with *T. phagedenis* strain 1498med (Genbank accession: KR025851.1) originally isolated from a dairy cow DD lesion in the USA (Walker et al., 1995).

An isolate (HY7) sharing 100% sequence identity similarity to the GI commensal treponeme OC1 (Genbank accession: GU566695.1) was isolated from a mucin cast sample. The second isolate, named HY5, from a mucin cast sample shared 100% sequence similarity with CHPA (Genbank accession: GU566699.1), a commensal of the ruminant GI tract which has recently been characterised as the new species *Treponema rectale* (Staton *et al*., 2017). Both mucin cast samples, from which an isolate was obtained, were positive by PCR for the *Treponema* genus. All isolation attempts failed from feed samples cultured; neither was spirochaete-like morphology observed in these cultures.

Whilst aiming to isolate treponemes, on occasion other anaerobic bacterial species isolates were obtained. From faecal samples two other bacterial isolates were obtained and shared 99% 16S rRNA sequence identity with *Victivallis vadensis* strain cello (Genbank accessions: NR_027565.1 and NR_118352.1). From mucin casts a bacterial isolate which shared 100% sequence identity to *Fusobacterium* sp. CLS-7530 (Genbank accession: EU597748.1) was

obtained along with two isolates sharing 94% and 95% sequence similarity to *Lachnospiraceace* bacterium oral taxon F15 strain UY038 (Genbank accession: HM099641.1). Furthermore there was also an isolate obtained which shared 98% sequence identity with an uncultured *Clostridiales* bacterium clone C073 (Genbank accession: EF434355.1). Bacterial species isolated which did not belong to the *Treponema* genus were not included in any further analysis.

Table 4.12: Statistical significance of healthy foot tissue PCR results[a]

	T. medium **presence n (%) [95% CI]**	***T. medium*** **absence n (%) [95% CI]**	***T. phagedenis*** **presence n (%) [95% CI]**	***T. phagedenis*** **absence n (%) [95% CI]**	***T. pedis*** **presence n (%) [95% CI]**	***T. pedis*** **absence n (%) [95% CI]**	**DD treponeme presence[b] n (%) [95% CI]**	**DD treponeme absence[b] n (%) [95% CI]**	***Treponema*** **presence[c] n (%) [95% CI]**	***Treponema*** **absence[c] n (%) [95% CI]**
Housing season Vs DD treponeme presence in healthy foot tissues										
Housing (*n*= 116)	11 (9.5) [4.8-16.3]	105 (90.5) [83.7-95.2]	17 (14.7) [8.8-22.4]	99 (85.3) [77.6-91.2]	4 (3.5) [0.9-8.6]	112 (96.6) [91.4-99.1]	18 (15.5) [9.5-23.4]	98 (84.5) [76.6-90.5]	68 (58.6) [49.1-67.7]	48 (41.4) [32.3-50.9]
Grazing (*n*= 101)	9 (8.9) [4.2-16.2]	92 (91.1) [83.8-95.8]	18 (17.8) [10.9-26.7]	83 (82.2) [73.3-89.1]	9 (8.9) [4.2-16.2]	92 (91.1) [83.8-95.8]	21 (20.8) [13.4-30.0]	80 (79.2) [70.0-86.6]	54 (53.5) [43.3-63.5]	47 (46.5) [36.5-56.7]
	P = 1.000		*P* = 0.581		*P* = 0.149		*P* = 0.376		*P* = 0.494	
Quarter of the year Vs DD treponemes in healthy foot tissues										
Jan-Mar (*n*=59)	9 (15.3) [7.2-27.0]	50 (84.8) [73.0-92.8]	13 (22.0) [12.3-34.7]	46 (78.0) [65.3-87.7]	3 (5.1) [1.1-14.1]	56 (94.9) [85.9-98.9]	14 (23.7) [13.6-36.6]	45 (76.3) [63.4-86.4]	36 (61.0) [47.4-73.5]	23 (39.0) [26.5-52.6]
Apr-Jun (*n*= 48)	6 (12.5) [4.7-25.2]	42 (87.5) [74.8-95.3]	10 (20.8) [10.5-35.0]	38 (79.2) [65.0-89.5]	6 (12.5) [4.7-25.2]	42 (87.5) [74.8-95.3]	13 (27.1) [15.3-41.8]	35 (72.9) [58.2-84.7]	27 (56.3) [41.2-70.5]	21 (43.8) [29.5-58.8]
July-Sept (*n*= 53)	3 (5.7) [1.2-15.7]	50 (94.3) [84.3-98.8]	8 (15.1) [6.7-27.6]	45 (84.9) [72.4-93.3]	3 (5.7) [1.2-15.7]	50 (94.3) [84.3-98.8]	8 (15.1) [6.7-27.6]	45 (84.9) [72.4-93.3]	27 (50.9) [36.8-64.9]	26 (49.1) [35.1-63.2]
Oct-Dec (*n*= 57)	2 (3.5) [0.4-12.1]	55 (96.5) [87.9-99.6]	4 (7.0) [1.9-17.0]	53 (93.0) [83.0-98.1]	1 (1.8) [0.02-9.4]	56 (98.3) [90.6-1.0]	4 (7.0) [1.9-17.0]	53 (93.0) [83.0-98.1]	32 (56.1) [42.4-69.3]	25 (43.9) [30.7-57.6]
	P = 0.102		*P* = 0.100		*P* = 0.164		*P* = 0.023		*P* = 0.765	

[a] *Associations were determined using Fisher's Exact test. The strength of association was determined using the adjusted critical P value of 0.01 and 95% CIs.*

[b] *Presence/absence of DD treponemes irrespective of which phylogroup.*

[c] *Presence/absence of Treponema genus (includes both commensal and pathogenic treponemes).*

Table 4.13: Statistical analysis of foot trimming equipment (including gloves) after trimming PCR results[a]

	T. medium **presence** **n (%) [95% CI]**	***T. medium*** **absence** **n (%) [95% CI]**	***T. phagedenis*** **presence** **n (%) [95% CI]**	***T. phagedenis*** **absence** **n (%) [95% CI]**	***T. pedis*** **presence** **n (%) [95% CI]**	***T. pedis*** **absence** **n (%) [95% CI]**	**DD treponeme presence[b]** **n (%) [95% CI]**	**DD treponeme absence[b]** **n (%) [95% CI]**
DD foot status Vs DD treponeme presence on all trimming equipment (including gloves) after trim								
DD-affected (*n* = 21)	4 (19.1) [5.4-41.9]	17 (81.0) [58.1-94.6]	11 (52.4) [29.8-74.3]	10 (47.6) [25.7-70.2]	0 (0.0) [-]	21 (100.0) [-]	11 (52.4) [29.8-74.3]	10 (47.6) [25.7-70.2]
DD-unaffected (*n*= 58)	1 (1.7) [0.04-9.2]	57 (98.2) [90.8-1.0]	2 (3.5) [0.4-11.9]	56 (96.6) [88.1-99.6]	0 (0.0) [-]	58 (100.0) [-]	2 (3.5) [0.4-11.9]	56 (96.6) [88.1-99.6]
	P = 0.016		*P* < 0.001*		-		*P* < 0.001*	
DD foot status Vs DD treponeme presence on trimming equipment only after trim								
DD-affected (n = 11)	0 (0.0) [-]	11 (100.0) [-]	2 (18.2) [2.3-51.8]	9 (81.8) [48.2-97.7]	0 (0.0) [-]	11 (100.0) [-]	2 (18.2) [2.3-51.8]	9 (81..8) [48.2-97.7]
DD-unaffected (*n* = 52)	1 (1.9) [0.0-10.3]	51 (98.1) [89.7-100.0]	2 (3.9) [0.5-13.2]	50 (96.2) [86.8-99.5]	0 (0.0) [-]	52 (100.0) [-]	2 (3.9) [0.5-13.2]	50 (96.2) [86.8-99.5]
	P = 1.000		*P* = 0.100		-		*P* = 0.100	
DD foot status Vs DD treponeme presence on gloves only after trim								
DD-affected (*n* = 10)	4 (40.0) [12.2-73.8]	6 (60.0) [26.2-87.8]	9 (90.0) [55.5-99.7]	1 (10) [0.3-44.5]	0 (0.0) [-]	10 (100.0) [-]	9 (90.0) [55.5-99.7]	1 (10.0) [0.3-44.5]
DD-unaffected (*n* = 6)	0 (0.0) [-]	6 (100.0) [-]	0 (0.0) [-]	6 (100.0) [-]	0 (0.0) [-]	6 (100.0) [-]	0 (0.0) [-]	6 (100.0) [-]
	P = 0.200		*P* = 0.001*		-		*P* = 0.001*	
DD foot status Vs DD treponeme presence on foot trimming equipment combined with Sullivan et al., (2014a) knife data after trim (cattle only)								
DD-affected (*n* = 28)	14 (50.0) [30.6-69.4]	14 (50.0) [30.6-69.4]	15 (53.6) [33.9-72.5]	13 (46.4) [27.5-66.1]	8 (28.6) [13.2-48.7]	20 (71.4) [51.3-86.8]	19 (67.9) [47.6-84.1]	9 (32.1) [15.9-52.4]
DD-unaffected (*n* = 59)	3 (5.1) [1.1-14.1]	56 (94.9) [85.9-98.9]	4 (6.8) [1.9-16.5]	55 (93.2) [83.5-98.1]	2 (3.4) [0.4-11.7]	57 (96.6) [88.3-99.6]	4 (6.8) [1.9-16.5]	55 (93.2) [83.5-98.1]
	P < 0.001*		*P* < 0.001*		*P* = 0.001*		*P* < 0.001*	

[a] *Associations were determined using Fisher's Exact test. The strength of association was determined using the adjusted critical P value of 0.013 and 95% CIs.*

[b] *Presence/absence of DD treponemes irrespective of which phylogroup.*

** Statistically significant association (P value < the critical P value of 0.013).*

Table 4.14: Statistical analysis of footprint PCR results[a]

	***T. medium* presence *n* (%) [95% CI]**	***T. medium* absence *n* (%) [95% CI]**	***T. phagedenis* presence *n* (%) [95% CI]**	***T. phagedenis* absence *n* (%) [95% CI]**	***T. pedis* presence *n* (%) [95% CI]**	***T. pedis* absence *n* (%) [95% CI]**	**DD treponeme presence[b] *n* (%) [95% CI]**	**DD treponeme absence[b] *n* (%) [95% CI]**
DD foot status Vs DD treponeme presence								
DD-affected (*n*= 89)	11 (12.4) [6.3-21.8]	78 (87.6) [79.0-93.7]	16 (18.0) [10.6-27.5]	73 (82.0) [72.5-89.4]	4 (4.5) [1.2-11.1]	85 (95.5) [88.9-98.8]	18 (20.2) [12.4-30.1]	71 (79.8) [69.9-87.6]
DD-unaffected (*n*= 80)	2 (2.5) [0.3-8.7]	78 (97.5) [91.3-99.7]	3 (3.8) [0.8-10.6]	77 (96.3) [89.4-99.2]	1 (1.3) [0.03-6.8]	79 (98.8) [93.2-1.0]	4 (5.0) [1.4-12.3]	76 (95.0) [87.7-98.6]
	P = 0.020		*P* = 0.003*		*P* = 0.400		*P* = 0.005*	
Floor surfaces Vs DD treponeme presence								
Rubber (*n*= 72)	7 (9.7) [4.0-19.0]	65 (90.3) [81.0-96.0]	9 (12.5) [5.9-22.4]	63 (87.5) [77.6-94.1]	2 (2.8) [0.3-9.7]	70 (97.2) [90.3-99.7]	11 (15.3) [7.9-25.7]	61 (84.7) [74.3-92.1]
Concrete (*n*= 53)	6 (11.3) [4.3-23.0]	47 (88.7) [77.0-95.7]	9 (17.0) [8.1-29.8]	44 (83.0) [70.2-91.9]	3 (5.7) [1.2-15.7]	50 (94.3) [84.3-98.8]	10 (18.9) [9.4-32.0]	43 (81.1) [68.0-90.6]
Metal (*n*= 44)	0 (0.0) [-]	44 (100.0) [-]	1 (2.3) [0.1-12.0]	43 (97.7) [88.0-99.9]	0 (0.0) [-]	44 (100.0) [-]	1 (2.3) [0.1-12.0]	43 (97.7) [88.0-99.9]
	P = 0.500		*P* = 0.047		*P* = 0.400		*P* = 0.020	

[a] *Associations were determined using Fisher's Exact test. The strength of association was determined using the adjusted critical P value of 0.013 and 95% CIs.*

[b] *Presence/absence of DD treponemes irrespective of which phylogroup.*

** Statistically significant association (P value < the critical P value of 0.013).*

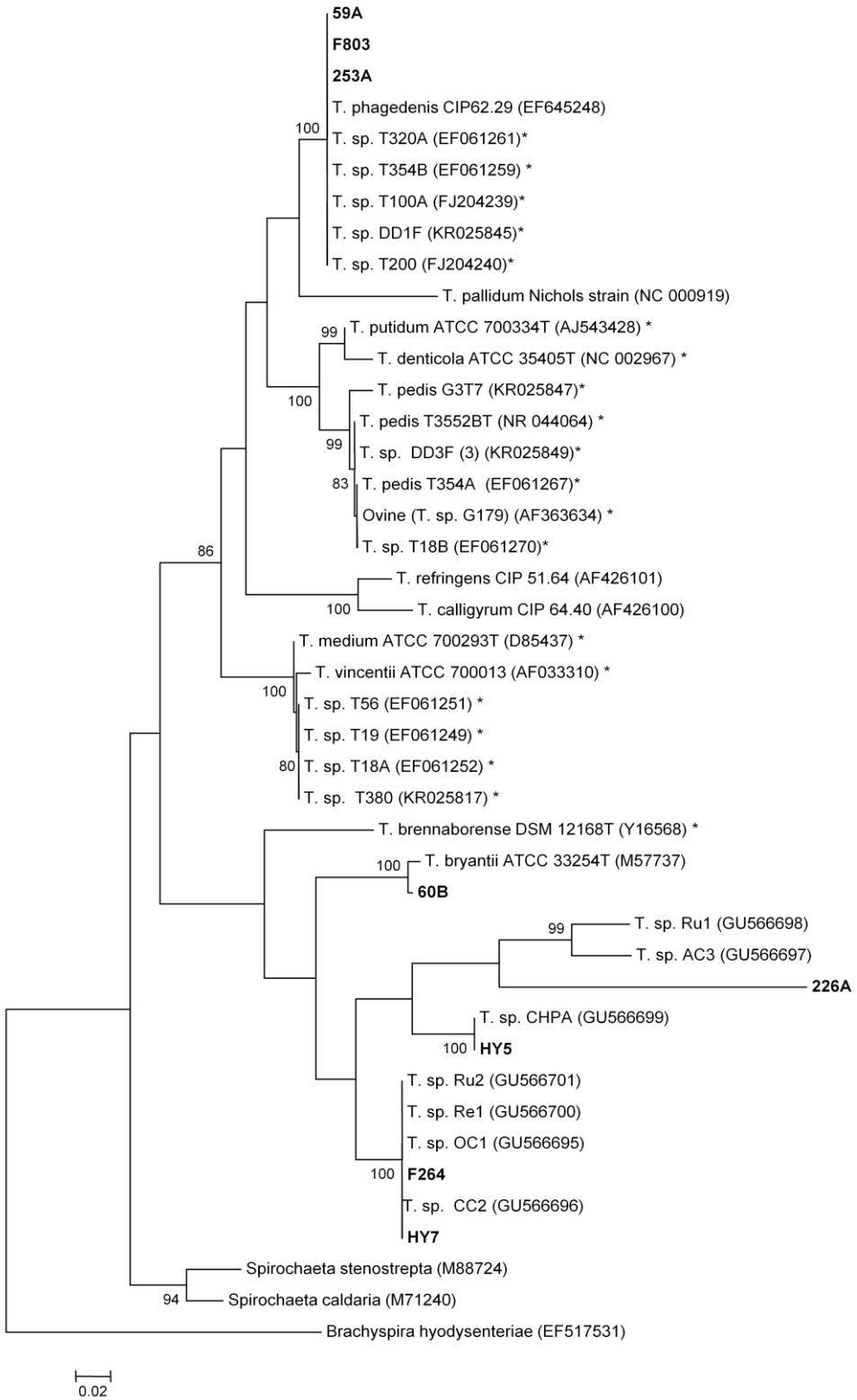


Figure 4.6: Phylogenetic tree of isolated treponemes from bovine tissues, bovine faecal and mucin cast samples based on an alignment of 16S rRNA gene sequences compared with other isolated treponemes.

*Comparisons of ~ 1000 aligned bases showing a relationship between strains isolated here (boldface type) from bovine gingiva and healthy foot tissues, bovine faces and bovine mucin casts and 16S rRNA genes from relevant isolated strains. Bootstrap confidence levels are shown as percentages of nodes (values of ≥ 70% are shown). * highlights 16S rRNA gene sequences previously reported from DD lesions. Genbank accession numbers are shown in parentheses.*

4.4 Discussion

This study aimed to further clarify the role of the cow and the dairy environment as infection reservoirs for DD by building upon previous work investigating dairy cattle gingiva, RAJ, healthy foot tissue, faeces and foot trimming equipment for DD treponeme presence as well as exploring new potential infection reservoirs including mucin casts, gloves, feed, water and footprints. Determining sites which are at risk of being a DD treponeme infection reservoir is key to understanding how transmission of the disease occurs and thus enables the development of strategies to prevent these transmission routes and progress towards elimination of DD from farms. Figure 4.7 shows a schematic of the different suspected infection reservoirs based on the identification of DD treponemes within them (combining the knowledge gained from this study with others studies) and how transmission may occur from them.

Following evidence that DD treponemes may reside in the bovine GI tract, particularly the gingiva and RAJ (Evans et al., 2012b), a larger scale survey was conducted to further understand this association resulting in the collection of gingival and RAJ tissue from a total of 122 cows. These tissues were sampled each month, over a two year period to ascertain whether temporal associations of DD treponemes with these tissue types occurred. DD treponemes were detected in sampled GI tract tissues using PCR, however, only in a small percentage of cows. Whereas previously, Evans et al., (2012b) identified DD treponemes from at least one of three cultivable DD treponeme phylogroups (*T. medium, T. phagedenis* and *T. pedis*) in gingiva and rectal tissue of 14.3% and 14.8% of cattle respectively; in this study DD treponemes were detected in only 6.6% and 0.8% of cattle gingiva and RAJ tissue respectively. The results from this study are more in-line with data collected by Sullivan et al., (2015a) who found DD treponemes to be present in 10% of beef cattle gingiva, 0% of beef cattle RAJ, 2.5% of sheep gingiva and 7.5% of sheep RAJ. However, this beef and sheep data was collected from a smaller proportion of animals (*n*=40) than in the current study

which may mean they are less likely to be a true representation of DD treponeme presence in the GI tract.

The small percentage of dairy cattle and other livestock surveyed positive for DD treponemes in either the gingiva or RAJ reaffirms that DD treponemes do not appear to be part of the normal microbiome found in these tissue types (Evans et al., 2012b; Sullivan et al., 2015a), which raises the question of why carriage occurs in some animals and not others? It may be that carriage is transient as described for *E. coli* O157 carriage in tissues of the lower GI tract of cattle (Naylor et al., 2003; Keen et al., 2010). Colonisation may also be associated with cow level factors such as immune response, physiological properties of the skin and diet (Somers et al., 2005; Palmer and O'Connell, 2015). Indeed, animals suffering from hind gut acidosis experience changes in the gut including epithelial damage and mucin shedding (Gressley et al., 2011), which may be a more favourable environment for DD treponeme colonisation in the RAJ. The presence of *mglB* in the DD treponeme genomes (unpublished genome data University of Liverpool; Becker et al., 1994) should enable mucin utilisation which taken together with recent investigations into DD treponeme invasion of other types of damaged tissues, for example cattle hock lesions (Svartström et al., 2013; Karlsson et al., 2014; Clegg et al., 2016a; c; d; e), is highly suggestive that DD treponemes may colonise the GI tract during tissue damage. Furthermore, during simulated subclinical acidosis of the rumen a recent study found that the relative abundance of *Treponema spp.* in the rumen increased by 9.6% (Petri et al., 2013). However, macroscopically in this study the GI tissues sampled appeared healthy.

Presence of DD treponemes in the gingiva could be an indicator for DD treponeme carriage in rumen fluid. In a previous whole cow survey treponemes belonging to the *T. phagedenis* DD treponeme phylogroup were detected in both the gingiva and rumen of one of the six cows investigated and were not detected in either of these tissues in any of the other cows (Evans et al., 2012b). Further investigation into gingiva carriage in conjunction with the rumen has not been carried out although investigations into each of these tissue types singly have been conducted (Evans et al., 2012b; Nascimento et al., 2015; Zinicola et al., 2015b). It may be that leakage of rumen fluid containing DD treponemes into the mouth may lead to their colonisation and subsequent detection in the gingiva or vice versa (Figure 4.7); this is also important as rumen fluid may also leak out of the mouth and provide a vehicle of transmission of DD treponemes to other cattle (Figure 4.7) (Nascimento et al., 2015). Additionally, although DD treponemes are only present in a small number of cattle

GI tracts, their role as an infection reservoir should not be discounted. It may be that these GI DD treponeme carrier cattle are super shedders or super spreaders, enabling a large amount of transmission despite the small number containing these infection reservoirs. Super shedding from the RAJ occurs in cattle colonised with *E. coli* O157, in which only a small proportion of cattle carrying *E. coli* O157: H7 in the RAJ shed high amounts of the bacteria into the environment (Low et al., 2005). Furthermore, another spirochaete, *Brachyspira hyodysenteriae* which causes swine dysentery in pigs can be maintained in pig populations by only a small number of carrier pigs (Songer and Harris, 1978; Duff et al., 2014). Therefore it may be that carriage of DD treponemes in the GI tract contributes to maintenance of DD infection on farms once the disease has been introduced or could in fact contribute to disease introduction.

Only one of the gingiva samples positive by PCR for DD treponemes (*T. phagedenis* DD treponeme phylogroup) was from a DD-affected cow which was similar to the results for beef cattle gingiva (Sullivan et al., 2015a). This is in contrast with the Evans et al., (2012b) survey which only detected DD treponemes in dairy cattle gingiva of those affected with DD. Conversely, Evans et al., (2012b) detected DD treponemes in the RAJ of DD affected and unaffected dairy cattle, whereas the DD treponeme positive RAJ obtained in this study was from a DD-affected cow and the sheep RAJ positive for DD treponemes in the Sullivan et al., (2015a) study were affected by CODD. Thus DD treponemes are not associated with GI tissue solely in DD-affected cows, suggesting presence in these different tissue types is independent of each other. This is worrying for DD control as cattle may be bought in that are DD-unaffected but carry DD treponemes in their GI tract thus acting as an unknown infection reservoir for DD if they are able to shed the DD treponemes into the environment.

Housing cattle has often been cited as a risk factor for DD (Somers et al., 2005; Onyiro et al., 2008), which has lead to an interest into whether DD infection reservoirs may be associated with the housing season. Evans et al., (2012b) observed that the majority of GI tissues positive for one or more of the *T. medium, T. phagedenis* and *T. pedis* DD treponeme phylogroups were detected from samples collected during the housing season (October-March) and determined that there was a statistically significant association between the detection of *T. phagedenis* DD treponeme phylogroup DNA in the rectal tissue and the housing season. With regards to sheep and beef cattle, DD treponemes were only detected in GI tissue sampled during the summer months with the majority of DD

treponemes detected in beef GI tract tissues collected on the same day (Sullivan et al., 2015a). This is in stark contrast to the previous study, however, sheep, beef and dairy cattle undergo different management practices which may result in differences in exposure and vulnerability to DD treponemes. It has also been surmised previously that as opposed to temporal associations being witnessed it could actually be small outbreaks of shedding on farm which may be independent of season, however, due to sampling methods it is unknown whether the positive GI tissue originated from the same or neighbouring farms (Sullivan et al., 2015a).

Temporal associations of DD treponemes with GI tract tissue was further investigated in this study with the larger sample population, nonetheless, in contrast to the two previous studies which saw distinct clustering of DD treponeme positive GI tissues with a particular season, there was no seasonal associations with DD treponeme GI tract carriage in this study. Although no association was observed and samples were spread out across the year suggesting there were no outbreaks of shedding either, this may not necessarily be the case. GI tissues were only sampled from a fallen stock yard for this survey; therefore although it is known these cows will have been situated in the North West of England, information about whether sampled cows came from the same or neighbouring farms is unknown. If GI tissues sampled from cattle were from different farms and different locations across North West England, it may be that a DD treponeme positive GI tissue came from a farm where an episode of DD treponeme GI carriage was occurring and as no other cattle sampled that day came from the same farm this association will have gone undetected. Secondly, carriage in the GI tissues may only be occurring on certain farms, without knowledge of farm origin, these associations may be missed. Finally, different dairy farms have different management practices with regards to housing, with some farms observing the housing and grazing seasons and others practicing zero-grazing. The housing practices of the cattle these tissues were sampled from were also unknown, therefore GI tissues PCR positive for DD treponemes outside of the housing season may actually be because these cattle are in zero-grazing systems. More detailed knowledge of the animals' background would be required to delineate DD treponeme temporal associations.

Whilst DD treponemes were detected in tissues by PCR, actual isolation of treponemes belonging to one or more of the *T. medium, T. phagedenis* and *T pedis* DD treponeme phylogroups from the GI tissue investigated in this study failed. Although it should be noted that spirochaete-like morphology was observed in cultures from twelve gingiva and eight

RAJ samples. DD treponemes can be incredibly difficult to isolate and maintain in culture due to their fastidious nature and are often out-competed by other bacteria present in the tissue when cultured. Isolation of DD treponemes from the GI tract would provide essential evidence that the treponemes are viable and thus have the potential to be transmitted in these tissue types. Excitingly, Sullivan et al., (2015a) was able to isolate a treponeme belonging to the *T. phagedenis* DD treponeme phylogroup from a CODD-affected sheep RAJ, thus giving evidence DD treponemes are viable in this niche and could potentially be transmitted from the RAJ.

The hind feet of surveyed dairy cattle, on the plantar aspect between the bulbs of the heel where DD lesions normally manifest, were also sampled for the presence of DD treponemes. Upon visual inspection the feet were classified as either 'healthy', 'DD lesion' or 'other'. The 12 DD lesions sampled during this survey were 100% positive for DD treponemes and despite the low number sampled, lesions showed similar associations of *T. medium, T. phagedenis* and *T. pedis* as described previously by Evans et al., (2009c). A total of 16 feet were classified as 'other' as they did not appear physically healthy but could not be definitively defined as having DD as described in Section 4.2.2. No photographs of feet were taken before sampling. However, this practice would have been useful to support descriptions of samples and in particular for 'other' feet sample classification. Of the 'other' feet, two feet were positive for DD treponemes with one of the feet positive for all three of the cultivable DD treponeme phylogroups. It may be that DD treponemes were opportunistically colonising less than healthy feet or perhaps were involved in the pathology. Indeed, DD treponemes have been described in other disorders of cattle feet (Evans et al., 2011a). A recent study described the presence of the *T. phagedenis* DD treponeme phylogroup in five out of eight irregular heel skin samples from dairy cattle in a DD disease-free herd and compared them to irregular heel skin from DD endemic herds in which all three DD treponeme phylogroups (*T. medium, T. phagedenis* and *T. pedis)* were identified (Luby et al., 2017). It was suggested that the presence of one DD treponeme phylogroup in the disease-free herd was insufficient to result in clinical DD (Luby et al., 2017). There was no description available of the irregular heel skin thus it is unknown whether they had a similar presentation to the 'other' feet described here, but the presence of DD treponemes in some samples but not all is similar to what was found in the this study. The lack of DD treponemes detected in the remaining feet in the current study suggests there is no DD lesion pathology occurring as DD treponemes are detected in 100% of DD lesions. The remaining 217 feet were described as healthy.

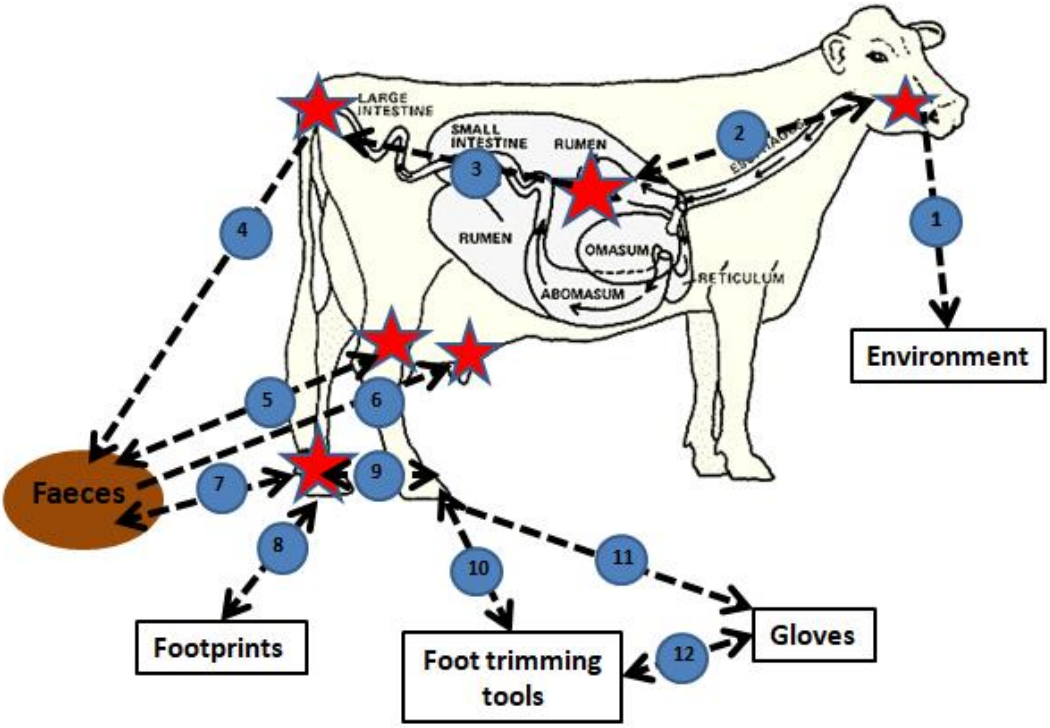


Figure 4.7: Schematic of how suspected DD treponeme infection reservoirs may contribute to transmission of DD

Infection reservoir sites in the dairy cow (red stars) identified in this study and other studies (from left to right, top to bottom) include the RAJ (this study and Evans et al., 2012b), the rumen including rumen fluid (Evans et al., 2012b; Nascimento et al., 2015; Zinicola et al., 2015b), the gingiva (this study and Evans et al., 2012b), healthy skin around the hock as well as disorders of the limb skin such as hock lesions and pressure sores (Evans et al., 2012b; Clegg et al., 2016a; d), ulcerative mammary dermatitis and ischaemic teat necrosis (Evans et al., 2010; Clegg et al., 2016c) and skin of the feet (with/ without DD lesions or non healing foot disorders) (this study, Choi et al., 1997; Stamm et al., 2002; Evans et al., 2008, 2009c, 2011a, 2012b; Klitgaard et al., 2008; Nordhoff et al., 2008; Luby et al., 2017). Other suspected infection reservoirs based on presence of DD treponemes in the schematic includes faeces, footprints (this study), gloves (this study and Angell et al., 2017) and foot trimming tools which encompass foot trimming knife blades (Sullivan et al., 2014a), hoof grinders (this study) and clippers (this study). Numbered dashed arrows indicate possible transmission routes for DD treponemes between suspected infection reservoirs. (1) DD treponemes in rumen fluid may leak out of the mouth during rumination and spread to the environment. (2,3) DD treponemes may pass through the GI tract enabling colonisation in the gingiva, rumen tissue/ fluid and the RAJ. (4) DD treponemes may be shed from the RAJ in faeces. (5) Faecal material in close contact with the limbs may enable DD treponeme transmission between faeces and either healthy skin, hock lesions or bovine pressures. (6) Faecal material in close contact with the udders/ teats may enable DD treponeme transmission between faeces and ischaemic teat necrosis lesions or areas of ulcerative mammary dermatitis on the udders. (7) Faecal material in close contact with the feet may enable DD treponemes to spread between faeces and skin with no visible DD lesions, DD lesions or non-healing foot disorders. (8) DD treponemes may be spread to floor surfaces in footprints made by cattle feet or vice versa. (9) DD treponemes may spread from foot to foot or skin to skin. (10) Foot trimming tools may become contaminated with DD treponemes from trimming feet with DD treponemes or vice versa. (11) Handling of feet infected with DD treponemes may result in DD treponemes

contaminating gloves and contaminated gloves may spread DD treponemes to feet handled. (12) Gloves contaminated with DD treponemes which handle trimming tools may then contaminate the trimming tools and vice versa. Figure adapted from (Sullivan, 2015).

A small number of healthy feet have often been used in studies to act as controls for the presence of DD treponemes when compared to DD lesions, although a small number of studies have detected DD treponemes in one or more healthy foot tissues (Strub et al., 2007; Evans et al., 2009c, 2012b). However, the Strub et al., (2007) study used broad ranging primers which may have detected more than just DD-associated treponemes. The detection of DD treponemes in healthy foot tissue prompted further investigation into the association of DD treponemes with the aforementioned large number of healthy foot skin tissues sampled in this survey. Interestingly, 18% of the healthy foot tissues sampled were positive for one or more of the *T. medium, T. phagedenis* and *T. pedis* DD treponeme phylogroups. In fact, the proportion of each of the three DD treponeme phylogroups detected in healthy foot tissue were similar to that of DD lesions (Evans et al., 2009c), with the *T. phagedenis* DD treponeme phylogroup representing the highest proportion of DD treponemes (16.1%) detected followed by the *T. medium* DD treponeme phylogroup (9.2%) and the *T. pedis* DD treponeme phylogroup (6%) with the least. The presence of DD treponemes in healthy foot skin raises interesting questions about the dynamics of DD treponeme infections; it may be that DD treponemes can be carried in healthy foot tissue without disease, the DD treponeme positive feet may have been recently infected and DD lesions are not macroscopically visible yet or that these feet may have previously suffered from DD and the DD treponeme infection has not been fully resolved which may result in later reoccurrence of the lesions.

In the previous studies, healthy foot tissue that were positive for DD treponemes either came from cows which presented with DD either on the same leg where the healthy tissue was taken or from another unaffected foot (Evans et al., 2012b), or were from a DD endemic farm (Evans et al., 2009c). Of the 39 healthy feet positive for DD treponemes collected in this study only five were from DD-affected animals, however, due to methods used for sampling in this study farm information is unknown and therefore it may be that the other DD treponeme positive healthy feet from DD unaffected cows were from DD endemic farms. DD treponemes have been shown to be detectable by PCR and culture on healthy skin a short distance from open hock lesions, another lesion in which DD treponemes have been associated, suggesting that DD treponemes may be able to survive

and migrate over the skin which may also aid in transference from one tissue site to the other (Figure 4.7) or from animal to animal and could explain presence on healthy foot skin demonstrated here (Clegg et al., 2016a). Furthermore, spirochaete-like morphology was observed in 14.7% of cultured healthy foot tissues in this study, with a couple of isolates obtained sharing 100% sequence similarity with strains from the *T. phagedenis* DD treponeme phylogroup. This indicates that the DD treponemes are alive and surviving on healthy skin and thus they may have the potential for transmission or DD lesion development.

When investigating the temporal associations between quarter of the year and the presence of DD treponemes on healthy feet there was weak evidence for an association between carriage of DD treponemes (regardless of phylogroup) on healthy foot tissue and quarter of the year they were sampled. However, there was no association with any individual DD treponeme phylogroup and quarter of the year. The largest numbers of healthy foot tissue were obtained in the months of January-March and April- June which incorporates the latter half of the typical housing season and the early half of the grazing season. It is difficult to speculate as to why this may be without more information about the farm practices the sampled cows took part in, however, it could be postulated that the increased incidence of DD when housed, along with close quarters and unhygienic conditions increases the level of DD treponemes in circulation meaning cattle are more likely to come into contact with the DD treponemes as the housing season draws on which result in greater detection towards the end of the housing season.

Whilst one DD treponeme positive gingiva sample and the only DD treponeme positive RAJ sample were from DD-affected cows it may also be that that the other cattle positive for DD treponemes in gingival tissue also have DD treponemes in healthy foot tissue. Indeed, two cattle with gingiva positive for DD treponemes had DD treponemes in at least one healthy foot. A cow who was positive for the *T. phagedenis* DD treponeme phylogroup in the gingiva was positive for all three DD treponeme phylogroups (*T. medium, T. phagedenis* and *T pedis*) in one healthy foot whereas the other cow was positive for the *T. medium* DD treponeme phylogroup in the gingiva and both hind healthy feet were positive for all three DD treponeme phylogroups investigated. It would be interesting to know whether the strains found in the GI tract of these cattle are the same as those found in the feet including for cattle in which a DD lesion is present. Zinicola et al., (2015b) used metagenomic sequencing to elucidate whether the same bacterial taxa were found in the

rumen, lesions and faeces of the same cows and found that *Treponema spp.* profiles were universal based on sequencing of a short region of the 16S rRNA gene.

Similar to previous studies no DD treponemes were detected in bovine faeces or mucin casts via a PCR approach despite using a DNA extraction method and DD treponeme phylogroup specific PCR assays previously optimised for use with DD treponemes in bovine faeces (Chapter 3) (Evans et al., 2012b; Sullivan et al., 2015a). Whilst the DNA extraction method and PCR assays were optimised for use with bovine faeces, it may be the dilutions of DD treponemes used to spike bovine faeces during optimisation was a gross overestimation of the actual amount of DD treponemes in field samples, as currently the exact quantities of DD treponemes in bovine faeces is unknown although current data suggests it to be only a very small proportion (<0.6%) of the bovine faecal/ slurry microbiota and that it may only be present in DD endemic herds (Klitgaard et al., 2014, 2017; Zinicola et al., 2015b). Studies which have been successful in detecting DD treponemes employ deep sequencing and shotgun metagenomic sequencing techniques which are much more sensitive than conventional PCR assays (Klitgaard et al., 2014, 2017; Zinicola et al., 2015b). Additionally, as the cattle in which faecal samples were taken were live it is unknown whether they have DD treponemes in their RAJ, with the low number of cattle carrying DD treponemes in the RAJ as described in this study and previously, it may be that faecal samples were not taken from RAJ carriers and are therefore unlikely to be shedding DD treponemes in faeces (Figure 4.7) (Evans et al., 2012b).

Remarkably, for the first time a DD treponeme, belonging to the *T. phagedenis* DD treponeme phylogroup, was isolated from faeces from a dairy cow which was not affected by DD but lived on a DD endemic farm. DD treponemes were not detected by PCR carried out on DNA extracted directly from this faecal sample. However, before culture enrichment the concentration of the DD treponemes within this faecal sample may have been below the limits of the detection for these assays or the PCR may have been affected by PCR inhibitors. This is the first evidence of viable DD treponemes in ruminant faeces, which would suggest DD treponemes are transmissible from faeces and further substantiates the role of bovine faeces as reservoir for DD (Figure 4.7).

Isolation of DD treponemes failed from all other faecal samples as well as mucin cast samples cultured, although spirochaete-like morphology was observed in 37.5% and 41.9% of mucin casts and faeces cultured respectively. However, two commensal treponemes OC1 and the recently named *T. rectale* were isolated from mucin casts as well as RU2 from

faeces (Staton et al., 2017). GI tract commensal treponemes have often been isolated from both bovine and ovine faeces and GI tract tissue and although they indicate that treponemes can survive and be shed in faeces they differ from DD treponemes in their morphology, growth characteristics and enzymatic activities forming their own grouping upon phylogenetic analysis away from DD treponemes (Evans et al., 2011b, 2012b; Sullivan et al., 2015a). Indeed, the *Treponema* genus PCR assay which detects both commensal and pathogenic treponemes was positive for 98.2% and 93.5% of faecal and mucin cast samples respectively, which is likely to be due to the GI tract commensal treponemes found in these sample types although may also be due to lesser characterised, non cultivated pathogenic treponemes.

Feed and water samples were also investigated as a reservoir for DD due to their link with the GI tract and possible method of DD treponeme entry into the GI tract. Furthermore feed samples were of particular interest due to preparation methods entailing anaerobic fermentation which could provide good conditions for treponemes to thrive and the associated changes in *Treponema* spp. levels in the rumen with dietary changes (Pitta et al., 2010; Petri et al., 2013). Such changes associated with diet may be due to nutrient changes affecting the growth of treponemes already in the rumen rather than the pathogens being introduction with feed. PCR and culture investigation of feed and water samples collected in this study failed to detect or isolate DD treponemes or the *Treponema* genus. Although extracted genomic DNA from feed was subsequently checked by a eubacterial 16S rRNA PCR assay to ensure nucleic acid extraction had been successful, the technique used for DNA extraction may not have been optimum for DD treponeme detection from this sample type, further investigation would be required to ensure the DD treponemes had the maximum chance of detection.

The detection of DD treponemes on foot trimming blades by Sullivan et al., (2014a) changed the way in which DD treponeme infection reservoirs were thought of, placing more emphasis on the role of fomites in transmission (Figure 4.7). In this study, DD treponemes were also detected on gloves and other trimming equipment used during routine foot trimming. DD treponemes were only detected on gloves following the trimming of DD-affected feet, which proved to be statistically significant, with only one DD-affected foot yielding no DD treponemes on gloves. As the gloves were changed between each cow and all fresh gloves tested were negative for DD treponemes, the DD treponemes must have transferred onto the gloves during the trimming of the DD-affected foot (Figure

4.7), most likely during cleaning and treatment of the lesions. The easy transference of treponemes through touch is highlighted by the *Treponema* genus PCR which detects both commensal and pathogenic treponemes. Only two fresh gloves prior to trimming were positive for treponemes whereas 100% of gloves sampled following trimming were positive for the *Treponema* genus. The presence of treponemes on two gloves prior to use may be due to contamination when gloves were changed. Although this survey was only carried out on a small number of samples these results are consistent with another study investigating the presence of DD treponemes on gloves following trimming of sheep affected and unaffected by CODD; whereby 100% of gloves from CODD-affected sheep were positive by PCR for one or more of the *T. medium, T. phagedenis* and *T. pedis* DD treponeme phylogroups and no DD treponemes were detected on gloves used to trim unaffected sheep (Angell et al., 2017).

Of the other foot trimming equipment (*n*=63) examined for DD treponeme presence, 6.3% were positive for one or more of the *T. medium, T. phagedenis* and *T. pedis* DD treponeme phylogroups. This foot trimming equipment consisted of powered hoof grinder discs and handles and clippers following dairy cow foot trimming; with one swab from a disc, one swab from a handle and two swabs from clippers positive for DD treponemes after use on different cows. Interestingly, unlike for the gloves, these samples containing DD treponeme DNA were not necessarily detected following the trimming of DD-affected feet, with the positive hoof grinder swabs obtained following trimming of DD-unaffected feet. Similarly in a previous study DD treponemes were present on foot trimming knives following trimming of both DD/CODD-affected and unaffected sheep and dairy cattle (Sullivan et al., 2014a). However, DD treponeme presence after contact with DD-unaffected feet may be linked to these animals residing in DD/CODD endemic farms and be either environmental contamination and/or very early non-visible lesions, especially as there were no DD treponemes detected on the hoof trimming knife following trimming at a dairy farm were DD was absent (Sullivan et al., 2014a). Furthermore, detection on DD-unaffected feet may be due to unaffected feet being carriers for DD treponemes following the detection of DD treponemes in healthy unaffected foot tissue in this study or possibly due to other non-healing afflictions of the hoof in which DD treponemes have previously been associated with (Evans et al., 2011a). However, when combined with glove data, there is a statistically significant association between the presence of DD treponemes on foot trimming equipment as a whole and the trimming of DD affected feet.

Here, for the first time DD treponemes have been detected in the footprints left behind by cattle standing in either the crush or the parlour. Crush and parlour floors were chosen for surveying footprints not only because it allowed ease for assessing feet for DD but cattle are confined to standing in the same spot for a period of time thus making treponeme transferral to the floor more likely. Furthermore cattle have limited space as to where they may place their hind feet thus increasing the likelihood that they may stand on or near to the location where the last cow stood. Thus the data presented here demonstrates DD treponeme positive footprints may provide a transmission route for DD from cow to cow (Figure 4.7). Furthermore there was a strong statistical association between the presence of DD treponemes in footprints and the footprints made by a DD-affected foot. Given the anatomical location of DD lesions this might be considered a surprising result with regards to lesion-floor contact. However, interestingly one DD-affected foot in which the footprint was positive for DD treponemes was observed to have the DD lesion touching the floor when stood in the parlour as shown in Figure 4.5b. An alternative route to floor surface contamination may be that the highly motile DD treponemes migrate out of the lesions and travel down the hoof (Clegg et al., 2016a). There was four DD-unaffected feet from four cows for which DD treponemes were detected in the footprints. Only one of these four cows had a DD lesion present on the other leg, which may have resulted in the transfer of DD treponemes to the unaffected leg. Other reasons for which DD treponemes may be present in footprints from DD-unaffected feet would be similar to those already described for foot trimming equipment and gloves.

Footprints were surveyed on three different floor types: metal, concrete and rubber for the presence of DD treponemes. The detection of DD treponemes on foot knife blades and periodontal disease treponemes on orthodontic metal brackets suggests that treponemes may be able to adhere to metal surfaces (Nelson-Filho et al., 2011; Andrucioli et al., 2012; Sullivan et al., 2014a). Therefore it could be hypothesised that DD treponemes are most likely to be found in footprints left on metal floors. However, of the three floor types surveyed, footprints on metal floors had the lowest DD treponeme detection rate of 2.3% compared to 18.9% and 15.3% of concrete and rubber floors respectively. Despite this difference in detection, there was not a significant association between floor type and detection. Investigations into foot-surface contact times with these different surfaces on farm, together with any changes in foot posture and *in vitro* mock contamination and decontamination of these surfaces are needed in the future to further dissect these

relationships. For example, whether time standing would affect DD treponeme presence in footprints is unknown and requires future investigation.

The type of floor surface used on dairy farms has come under scrutiny in various studies for associations with lameness and claw disorders; including DD (Wells et al., 1999; Cook, 2003; Somers et al., 2003; Frankena et al., 2009). Concrete is the most predominant floor material used in dairy systems, which is worrying when examining the footprint data in this study as concrete has the highest detection rate when compared with metal and rubber floors for DD treponemes in footprints. The hard, slippery and abrasive nature of concrete floors as well as the ability of slurry to become trapped in certain types of concrete floor designs such as 'grooved' have been cited as possible reasons for the increased risk of claw disorders and DD observed in these types of housing systems (Wells et al., 1999; Somers et al., 2005; Barker et al., 2009; Frankena et al., 2009). The presence of DD treponemes on concrete floors from footprints further adds to this risk as not only do DD treponeme footprints potentially provide an infection reservoir for DD on this floor type but abrasion suffered by feet from the concrete could be postulated to provide an entrance route for infection. In fact, infection models of DD have demonstrated that abrasion of the skin on the plantar aspect of the foot between the bulbs of the heel is required in most cases for DD lesion development (Gomez et al., 2012; Krull et al., 2016). Many farms are moving towards using more rubber for floor surfacing in walkways and the parlour due to reported health benefits including reduced risk for claw disorders including DD compared to concrete flooring (Vokey et al., 2001; Telezhenko et al., 2007; Ouweltjes et al., 2009; Fjeldaas et al., 2011; Eicher et al., 2013). Whilst in this study DD treponemes have been detected in footprints on rubber floors, and only moderately less than concrete (15.3% and 18.9% respectively), it could be argued that softer surfaces result in less damage to feet and thus there is less opportunity for DD treponemes to gain entry. However, the presence of DD treponemes within footprints on rubber floors is still cause for concern.

The detection of DD treponemes on gloves, foot trimming equipment and in floor footprints via PCR provides evidence that fomites that come into close contact with the foot may be a possible transmission route for DD (Figure 4.7), but transmission is dependent on the DD treponemes being viable which was not investigated in this study. DD treponemes are notoriously difficult to culture and isolate, however, treponemes belonging to the *T. phagedenis* DD treponeme phylogroup was successfully grown and isolated from a foot knife (Sullivan et al., 2014a). Furthermore, Angell et al., (2017) successfully cultured

DD treponemes from swabbed gloves demonstrating that the treponemes are viable and thus transmissible (Angell et al., 2017). Additionally DD treponemes belonging to the *T. phagedenis* and *T. pedis* DD treponeme phylogroups were able to remain viable on gloves in aerobic conditions for up to 3 days (Angell et al., 2017). The ability of DD treponemes to survive aerobically for 3 days suggests they may not be obligate anaerobes as previously described. Indeed there have been other similar anecdotal accounts. Whether DD treponemes form a biofilm which enables survival under aerobic conditions or are indeed facultative anaerobes requires further study. Further studies would also be required to ascertain whether DD treponemes in footprints and other trimming equipment are viable and for how long for, however, the current evidence does suggest it is highly likely these DD treponemes are viable and thus transmissible.

Interestingly, for almost all sample types except gingiva surveyed in this study, DNA from the *T. phagedenis* DD treponeme phylogroup was the most readily detected of the three cultivable DD treponeme phylogroups studied in the UK. It has also been the only DD treponeme phylogroup isolated from foot tissues and faeces in this study. What is more, the proportion of samples positive for the *T. medium, T. phagedenis and T. pedis* DD treponeme phylogroups for sample types including footprints, healthy feet, gloves and trimming equipment is reflective of the general proportion of these phylogroups observed in the DD lesions themselves with *T. phagedenis* DD treponeme phylogroup the most prominent and in the highest percentage of DD lesions followed closely by the *T. medium* DD treponeme phylogroup and finally the *T. pedis* DD treponeme phylogroup (Evans et al., 2009c). Although in some sample types, especially fomites, the *T. pedis* DD treponeme phylogroup was not detected at all. Using the same PCR approach similar DD treponeme phylogroup associations have also been reported in beef DD lesions (*T. medium*: 79%, *T. phagedenis*: 91%, *T. pedis*: 71%) (Sullivan et al., 2015c), CODD lesions (*T. medium*: 67%, *T. phagedenis*: 85%, *T. pedis*: 71%)(Sullivan et al., 2015c) and to some extent hock lesions (*T. medium*: 47%, *T. phagedenis*: 100%, *T. pedis*: 47%) (Clegg et al., 2016a).

Whilst these DD treponeme phylogroup detection rates in different sample types could be explained by sensitivity of the DD treponeme phylogroup specific PCR assays, it is unlikely to be the case because although sensitivity is highest for the *T. phagedenis* DD treponeme phylogroup it is the *T. medium* DD treponeme phylogroup PCR assay which has the lowest sensitivity of the three PCR assays not the *T. pedis* DD treponeme phylogroups (Evans et al., 2009c). Furthermore, these DD treponeme phylogroup proportions have not been

demonstrated in all sample types such as the GI tract in this study as well as in other studies, for example, caprine DD lesions and foot trimming knife blades in which the *T. medium* DD treponeme phylogroup dominated (Evans et al., 2012b; Sullivan et al., 2014a, 2015a; Crosby-Durrani et al., 2016). The fact that similar proportions of DD treponeme phylogroups observed in lesions are also observed in footprints, healthy hind feet and other equipment reiterates that DD treponemes may be transferred to these sample types by direct contact with lesions which may account for why similar phylogroup associations are detected.

The increasing body of evidence for the presence of DD treponemes on fomites that readily come into contact with the foot is of great concern when considering control and prevention of DD/CODD. DD treponeme presence on foot trimming equipment, gloves and crush floors corroborates with previous studies that have cited foot trimming practices as a risk factor for DD (Wells et al., 1999; Holzhauer et al., 2006). Indeed, these fomites could readily pass DD treponemes from one cow to another through direct contact if foot trimming equipment or the crush/ parlour floor is contaminated with DD treponemes from the previous cow (Figure 4.7). This may also be possible in footprints made on walkways although this has not yet been surveyed. Furthermore, evidence of DD treponeme viability as long as 3 days on the gloves (Angell et al., 2017) is worrying for between farm transmission, especially if similar viability is seen on other equipment including the footprints on the crush floor as many foot trimmers bring their own crush on farm.

Disinfection of equipment and floors between cows and farms may be one way to control these potential infection reservoirs and prevent transmission. There have been some investigations into efficacy of different disinfection methods for DD treponemes on equipment. In this study plain water collected from a nearby trough was used to clean clippers following trimming of each cow. Detection of the *Treponema* genus, that was originally detected by PCR on 100% of clippers (*n*=18), was reduced to zero. In addition, following washing DD treponemes were no longer detected on the clippers that had tested positive for DD treponemes prior to washing (*n*=2). However, further investigation is required with a greater number of DD treponeme positive clippers to discern this methods efficacy against DD treponemes. Sullivan et al., (2014a) used a DEFRA-approved iodine (2.5% w/v) disinfectant (no brand specified) to disinfect foot trimming knife blades following use. DD treponeme PCR detection rates dropped considerably on knife blades following disinfection but disinfecting did not completely stop detection. For example,

detection of the *T. pedis* DD treponeme phylogroup reduced from 54% of blades PCR positive to 8% of blade PCR positive (Sullivan et al., 2014a). With this study, the viability of the DD treponemes detected on blades following disinfection is unknown and further studies are required to follow this up. Furthermore further study would be required into the viability of DD treponemes in footprints and whether disinfectants are effective; taking into consideration contact times and concentrations of active compounds. Interestingly cleaning gloves with disinfectants of either hand soap (with water), 1% Virkon, 1:90 dilution of FAM or 70% ethanol resulted in no growth of DD treponemes in culture and vastly reduced detection by PCR (Angell et al., 2017). However, washing gloves with warm or cold water was ineffective in preventing detection of DD treponemes by PCR and culture (Angell et al., 2017). Clearly further study using a larger DD treponeme positive sample population is required to determine which disinfectant, disinfectant concentration and disinfection procedure will completely disinfect equipment from DD treponemes and be practical in farming practice.

In conclusion, a lower percentage of dairy cow GI tract tissues were positive for DD treponemes than previously reported following a larger sample size survey. However, without further investigation into the epidemiology of GI tissue carriage the significance of the GI tract positive cattle in terms of an infection reservoir cannot be known. Interestingly, DD treponemes have been detected by PCR and isolation in a number of healthy feet where DD lesions would normally be located, raising the question as to whether this is as a result of new infection, old resolved infection or carriage without causing changes to the healthy tissue. A DD treponeme was isolated from a bovine faecal sample, providing evidence for the first time of DD treponeme viability in faeces and further supporting the role of faeces as a DD infection reservoir. Additionally, for the first time DD treponemes have been detected in the footprints left behind on different floor types in the parlour and crush. Furthermore disposable nitrile gloves and a small number of foot trimming equipment including hoof grinders and clippers have tested positive for DD treponemes. This adds to a growing body of evidence that suggests fomites may be important infection reservoirs for DD transmission between cows and farms. It is therefore imperative that hygiene measures such as disinfection are further investigated to identify a practical effective method for the removal of DD treponemes from such fomites to reduce DD.

Chapter 5: Characterisation of DD treponeme carriage

5.1 Introduction

The detection of DD-associated treponemes in dairy cattle non-DD lesional tissues such as healthy feet, gingiva and RAJ, as described by Evans et al., (2012b) and in this thesis (Chapter 4), raises important questions about the carriage of DD treponemes in these tissue types, not least because these tissues appear macroscopically healthy. Whilst DD is considered a polymicrobial disease, *Treponema* is the only genus of bacteria consistently identified in DD lesions. In the UK and USA, the three cultivable phylogroups of DD treponemes: *T. medium, T. phagedenis* and the recently named *T. pedis,* are identified in one hundred percent of lesions and are therefore considered integral to DD pathogenesis (Stamm et al., 2002; Evans et al., 2008, 2009b; c). Only in recent years have these DD treponeme phylogroups been detected in other tissue types to DD lesions in cattle and other livestock during the pursuit of infection reservoirs (Evans et al., 2012b; Nascimento et al., 2015; Sullivan et al., 2015a; Zinicola et al., 2015b; Clegg et al., 2016a; c; d; e). Hence, thus far there has been limited investigation into DD treponeme carriage.

Whether DD treponemes can be carried in healthy tissue without evidence of tissue abnormalities and where in the tissue the DD treponemes localise requires investigation. Methods involving histopathology and immunohistochemistry (IHC) have frequently been used in DD and CODD studies to describe the active disease process (Dopfer et al., 1997; Cruz et al., 2005; Evans et al., 2009c; Rasmussen et al., 2012; Angell et al., 2015b). In the region of the foot where DD lesions are usually found in cattle, on the plantar aspect of the pastern, between the bulbs of the heel, the skin is similar in structure to elsewhere on the cow with the exception that it is usually thickened, with the presence of retes pegs and as the skin progresses down towards the coronet, the frequency of hair follicles decreases (Budras et al., 2003). Figure 5.1 shows an example of the structure and cell layers which form the bovine skin. Upon histopathological evaluation, it is normal for healthy skin in this region to have a uniform smooth stratum corneum and mild perivascular infiltrates of lymphocytes and plasma cells in the dermis (Dopfer et al., 1997). Studies investigating the histopathology of DD lesions in cattle find abnormalities in the tissues with typical changes

including overall thickened epidermis, loss of the epidermis (ulceration), irregular stratum corneum with erosion, parakeratotic and/or orthokeratotic hyperkeratosis, acanthosis, pronounced retes ridge formation with microabscesses present at the tips of the dermal papillae, invasive bacteria, haemorrhaging, cell debris and necrotic tissue, large perivascular infiltrates of mononuclear cells (lymphocytes and monocytes) in the dermis with increasing levels of neutrophils and eosinophils in the dermis and epidermis (Blowey et al., 1994a; Dopfer et al., 1997; Manske et al., 2002; Cruz et al., 2005; Klitgaard et al., 2008; Berry et al., 2010; Rasmussen et al., 2012; Knappe-Poindecker et al., 2013; Krull et al., 2014; Nielsen et al., 2016). Different DD lesion stages have different levels of abnormalities in the tissue, with thickness of the epidermis generally increasing with each stage, reaching its peak during chronic stage (M4) DD lesions, whereas ulceration of the epidermis, characterised by the complete loss of the epidermis, is a key feature of 'classic' (M2) DD lesions (Dopfer et al., 1997).

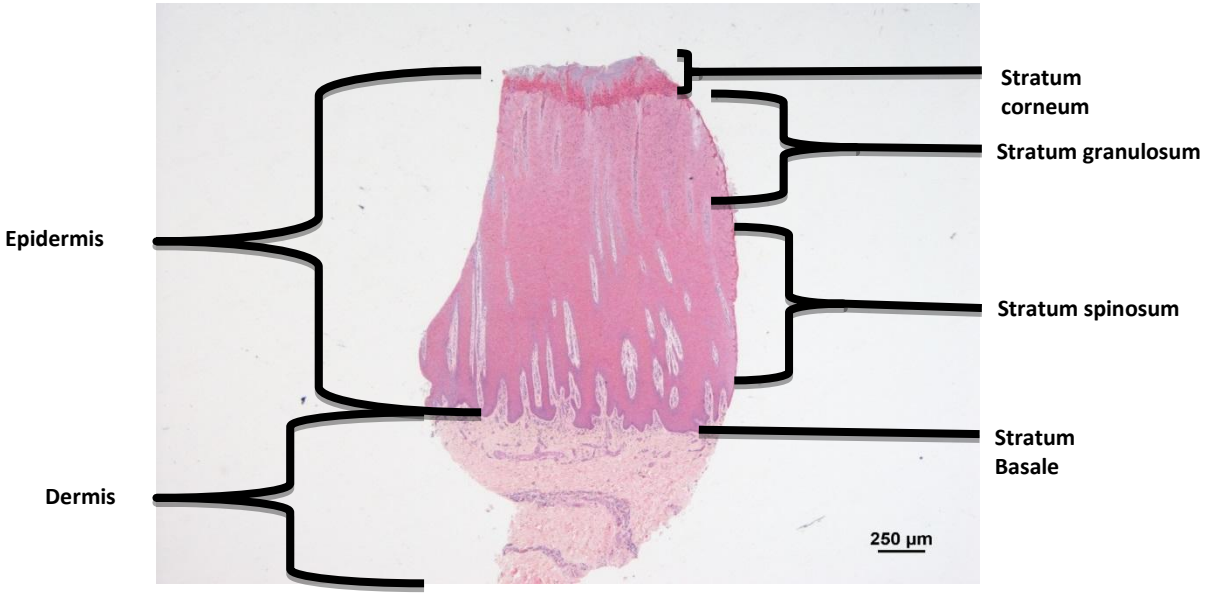


Figure 5.1: Example of bovine foot skin anatomy

Haematoxylin and eosin stained section of skin from what is considered a sub-clinical DD lesion from this study, from between the bulbs of the heel on the plantar aspect of the bovine foot collected during sampling for this thesis. The epidermis is severely thickened and the stratum corneum is irregularly eroded. Photograph and labelling credit: Dr Hayley Crosby-Durrani, resident pathologist at the School of Veterinary Science, University of Liverpool, UK.

Various techniques, including silver staining methods, fluorescence *in situ* hybridisation (FISH) and IHC have enabled visualisation of spirochaetes and more specifically treponemes

within the tissues; allowing studies to determine their localisation within DD lesions (Dopfer et al., 1997; Demirkan et al., 1998; Cruz et al., 2005; Klitgaard et al., 2008; Evans et al., 2009c; Rasmussen et al., 2012; Nielsen et al., 2016). Spirochaetes are observed as the most abundant type of bacteria within DD lesions when using these techniques (Blowey et al., 1994b; Demirkan et al., 1998; Cruz et al., 2005; Klitgaard et al., 2008; Nielsen et al., 2016); an observation which has been corroborated by metagenomic studies investigating DD lesion microbiomes (Klitgaard et al., 2014; Krull et al., 2014; Zinicola et al., 2015b). In bovine DD lesions, spirochaetes have been shown to localise either in the upper surface layers of the skin and/ or deep within the skin in the intercellular junctions between the cells, particularly in the stratum spinosum and spreading as far as the dermis (Dopfer et al., 1997; Moter et al., 1998; Cruz et al., 2005; Klitgaard et al., 2008; Evans et al., 2009c). Spirochaetes appear to further localise in areas where abnormalities in the tissue are prominent. Dopfer et al., (1997) describes spirochaetes localising along the horny columns that form during DD and short spiral microorganisms presumed to be spirochaetes at the sites of ballooning degeneration and Klitgaard et al., (2008) observed DD treponemes in areas of keratinolysis, between degenerated keratinocytes and within lysed keratinocytes. Evans et al., (2009c) detected strong labelling for treponemes, using IHC, in the sebaceous glands and hair follicles of DD lesions; which implies that these structures could provide a means for the treponemes to penetrate past the initial physical barrier of the skin and into the deeper layers of the epidermis where infection can then be established and maintained to enable chronic DD.

Studies have applied staining/ labelling techniques to healthy foot tissues which have no histopathological changes, mostly to act as controls for comparison with DD lesions. Spirochaetes/ treponemes appear to be scarcely found in healthy foot tissue, with no silver staining or fluorescent (FISH) or IHC labelling occurring within the skin (Dopfer et al., 1997; Cruz et al., 2005; Evans et al., 2009c; Nielsen et al., 2016); with the exception of one study which picked up fluorescence for spiral type microorganisms (not definitively spirochaetes) in healthy controls but levels were significantly less than what would be seen in DD lesions (Dopfer et al., 1997).

In humans, *Treponema denticola,* which clusters phylogenetically with the *T. pedis* DD treponeme phylogroup, is one of the main bacterial species associated with periodontal disease (a chronic inflammatory disease of the gingiva)(Choi et al., 1994). Likewise *Treponema vincentii*, which is closely related to the *T. medium* DD treponeme phylogroup is

also implicated in human periodontal disease (Choi et al., 1994). Treponemes have also recently been detected by PCR in 70% of samples taken from a type of periodontal disease in horses; the majority of treponemes detected had sequences similar to *T. pectinovorum* (another reported human oral treponeme)*, T. denticola and T. medium* (Sykora et al., 2014). However, in the aforementioned study, some unaffected oral horse tissues contained *Treponema* spp. suggesting these bacteria may actually be part of the normal microbiome (Sykora et al., 2014; Gao et al., 2015). Most recently *T. medium and T. denticola* have also been identified in ovine periodontal disease (Borsanelli et al., 2017).

Whilst, one or more of the *T. medium, T. phagedenis and T. pedis* DD treponeme phylogroups have been detected at the gingiva in dairy cattle (as well as beef cattle and sheep) (Evans et al., 2012b; Sullivan et al., 2015a), there was no indication that these samples came from cows with periodontal disease. Thus far, DD treponeme phylogroups have not been linked with bovine periodontal disease although *T. denticola* has been (Borsanelli et al., 2015). However, in light of recent DD treponeme detection in the oral cavity, it is yet to be determined if DD treponemes are carried in the bovine gingiva without disease or whether there are underlying microscopic changes in the mucosa that could be associated with disease. Although presence of DD treponemes with abnormalities in the tissue does not necessarily determine cause, it may be that damaged tissue enables their colonisation of these non-pedal tissues. Should abnormalities be present in the gingival tissue where DD treponemes have been detected in this thesis, based on periodontal disease, it could be expected that general changes such as the formation of pockets in the gingiva, ulceration of the epithelium, severe infiltrates of lymphocytes, neutrophils macrophages and plasma cells, degradation of periodontal attachments, increased vascular supply and proliferation of epithelium may be observed (Cox et al., 2012; Hasan and Palmer, 2014).

Previously, studies have been able to detect the *Treponema* genus and isolate commensal (non-pathogenic) treponemes from bovine rectal tissue (Evans et al., 2012b; Mao et al., 2015; Zaheer et al., 2017); which had prompted further investigation into the role of this tissue as a potential reservoir for pathogenic DD treponemes. Recently, two studies and a study in this thesis (Chapter 4 section 4.3.1.1) have identified one or more of the DD treponeme phylogroups in the recto-anal junction (RAJ) (Evans et al., 2012b; Sullivan et al., 2015), with one study demonstrating that the DD treponemes are likely to be viable in the RAJ and thus could be transmissible (Sullivan et al., 2015). The RAJ comprises of stratified

squamous epithelial cells that form the anus and transitions into columnar epithelial cells of the mucosa of the rectum (Tanaka et al., 2012). *Escherichia coli* O157 colonisation of the RAJ in cattle does not outwardly appear to produce disease (Nart et al., 2008). However, histopathological changes have been observed at sites of colonisation (Nart et al., 2008). Furthermore, a concurrent DD and enteritis of cattle has been reported in Japan with IHC implicating spirochaetes in both (Shibahara et al., 2002). Taken together, as with the gingiva, DD treponeme colonisation in the RAJ requires further investigation to determine whether carriage is associated with abnormalities in the tissue and whether the spirochaetes have a specific cellular localisation or tropism.

Multi locus sequence typing (MLST) is a genotyping technique which enables further phylogenetic characterisation of bacteria through analyses of sequence differences present between isolates at seven relevant housekeeping genes (Maiden et al., 1998). These differences in sequence are designated as different alleles for each of the housekeeping genes which results in an allelic profile for each isolate known as a sequence type (ST). MLST was recently utilised to further characterise DD lesion isolates from the *T. medium*, *T. phagedenis* and *T. pedis* DD treponeme phylogroups, isolated from different species and geographic areas, to determine if certain strains were host and/or geographically specific in light of recent discoveries of DD manifestations in new species (Clegg et al., 2016b). This treponeme MLST demonstrated that there was generally a low level of diversity within DD phylogroups and that STs were not confined to a particular geographic location or species although similar ST profiles were observed circulating between animals on farm and interestingly between different animal types in a similar geographic region (Clegg et al., 2016b).

In terms of further understanding pathogen carriage, MLST helped to determine, for the well-defined human pathogen *Neisseria meningitidis,* that there are a variety of meningococci carried in the nasopharynx whilst only a proportion of hyperinvasive lineages are actually responsible for disease (Yazdankhah et al., 2004). More recently, MLST has been applied to a dairy cattle mastitis pathogen to delineate that a small number of *Streptococcus uberis* STs are extremely important for disease epidemiology; implicating cow to cow transmission instead of the environment as most important in the UK (Davies et al., 2016). Whilst the aforementioned DD treponeme MLST study focused on geographical and species distribution of DD treponeme STs, these investigations could now be applied to a more local level by investigating DD treponeme phylogroup STs present

within individual cattle. Although DD treponeme phylogroups (*T. medium, T. phagedenis* and *T. pedis*) appear to colonise different tissue types, in different anatomical locations and in different species, MLST would delineate whether the same ST types within a DD treponeme phylogroup are able to infect gingiva, RAJ and foot tissue or whether there is specific tissue tropism among STs. Furthermore, where a single cow has DD treponemes of the same phylogroup present in multiple sites, MLST would help to determine whether it is the same ST present at all sites and therefore considered the same strain. The knowledge gained would provide insight into the role of DD treponemes in the GI tract as an infection reservoir for DD.

The aims of this study are to further characterise the carriage of DD treponemes in healthy tissue by: 1) Determining the relationship between DD treponemes and healthy tissues positive for DD treponemes by PCR using histopathology and IHC to visualise whether there are any abnormalities in the tissue, if DD treponemes are visible and where in the tissue the DD treponemes localise. 2) To use MLST to establish whether the same DD treponeme phylogroup STs can be found in different tissues and within the same cow.

5.2 Materials and methods

5.2.1 Antigen preparation of commensal treponemes

From archived culture stocks, two strains of GI commensal treponemes RU1 and CHPA which were recently designated as newly named species *T. ruminis and T. rectale* respectively (Newbrook et al., 2017; Staton et al., 2017), were inoculated and cultured as described in Chapter 2, Section 2.5.3. Cultures were checked and sub-cultured every 1-2 days as per the growth requirements of GI commensal treponemes (Evans et al., 2011b; Newbrook et al., 2017; Staton et al., 2017). Once optimal levels of growth were achieved, the cultures were transferred from the anaerobic cabinet to the laminar flow for antigen preparation.

Of each treponeme culture, 10 ml was centrifuged at 10 000 *g* at 20°C for 30 minutes. The supernatant was then discarded. Next, 5 ml of 5mM $MgCl_2$ (Chapter 2, Table 2.2) was added to each cell pellet and vortexed; this step was repeated. The cell pellet resuspended in $MgCl_2$ was then centrifuged at 10 000 *g* at 20°C for 30 minutes and the supernatant discarded. The above steps were repeated once more with 5 ml of 5 mM $MgCl_2$ added to each pellet, vortexed to mix and repeated. The suspended cell pellets in $MgCl_2$ were then centrifuged again at 10 000 *g* at 20°C for 30 minutes. Cell pellets were resuspended in 1 ml

1X PBS (Chapter 2, Table 2.2). Resuspended cell pellets were sonicated on ice for 30 seconds followed by resting on ice for 20 seconds; the sonication process was repeated four times. Subsequently, 20 µl of Nonidet P-40 (Sigma-Aldrich, Dorset, UK) and 10 µl of 100mM EGTA (Chapter 2, Table 2.3) were added to each sonicated supernatant and then incubated at 37°C for 4 hours, occasionally mixing via inversion during incubation. Following incubation, the supernatants were frozen at -20°C for 60 minutes. Once thawed, the supernatants were centrifuged at 20°C for 15 minutes followed by dialysis against a litre of 1X PBS using 12-14 kDa 6.3 mm thickness visking tubing for 72 hours at 4°C. The PBS dialysis liquid was changed every 8 hours. The protein concentration of the dialysed antigen was measured via the Nanodrop 2000 spectrometer (Thermo Scientific, Hemel Hempstead, UK) and then aliquoted and stored at -20 °C.

5.2.2 One-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (1D SDS-PAGE) of gastrointestinal commensal treponemes

For 1D SDS-PAGE, two 12% (v/v) resolving SDS-polyacrylamide gels were prepared as described in Chapter 2, Table 2.2 and polymerized within a mini-gel system (Mini-PROTEAN electrophoresis system, Bio-Rad, Hemel Hempstead, UK) containing glass plates (0.75 mm short plates and spacer plates) and gel cassettes (Bio-Rad, Hemel Hempstead, UK). Gels were overlayed with isopropanol (Bio-Rad, Hemel Hempstead, UK) and allowed to polymerise for approximately 30 minutes. Next, 5% (v/v) SDS-polyacrylamide stacking gels were prepared as described in Chapter 2, Table 2.2. Once prepared, the stacking gel was immediately poured on top of the resolving gel and 15 lane Teflon combs (mini protean combs 0.75 mm, Bio-Rad, Hemel Hempstead, UK) were inserted to produce wells in the gel. Gels were allowed to polymerise for 30 minutes before the combs were removed and the gels were washed with ddH20 to remove excess acrylamide. Once the gels were cast, they were transferred to an electrophoresis tank (Bio-Rad, Hemel Hempstead, UK) and submerged in 1X Tris-glycine running buffer (Chapter 2, Table 2.3).

Antigen preparations of GI commensal treponemes *T. ruminis* and *T. rectale* prepared in Section 2.4.9 and an antigen preparation (sonicated and detergent extracted) of DD treponeme phylogroup *T. medium* strain T19 (prepared previously by Jenna Lowe, a member of the Infection Biology technical team at University of Liverpool), were diluted to a 5 mg/ ml concentration. Next, 80 µl of each antigen preparation was added to 20µl of 5X SDS gel-loading sample buffer (Bio-Rad, Hemel Hempstead, UK) and dissolved by heating at 100°C for 3 minutes. Then 5 µl of protein ladder (P7711S Colorplus Prestained Protein

ladder broad range 10-230 kDa) (NEB, Hertfordshire, UK) was loaded onto the gel followed by 10 µl of each treponeme antigen preparation. Gels were run in the electrophoresis tank using a Bio-Rad Powerpac 300 (Bio-Rad, Hemel Hempstead) at 180 V, 400 mA for 50 minutes.

5.2.3 Western blot to determine binding of anti-treponemal antibodies to commensal treponemes compared to DD treponemes

Rabbit polyclonal anti-treponemal antibodies used in this study had previously been prepared as described by Evans et al., (2009c). Briefly, antigen preparations from each of the three DD treponeme phylogroups (*T. medium, T. phagedenis* and *T. pedis*) were pooled and inoculated into rabbits with Fruend's complete and incomplete adjuvants via a multisite regimen over 3 months by a commercial concern (Evans et al., 2009c). Polyclonal antibodies that cross reacted with the three DD treponeme phylogroups were obtained at the terminal bleed.

Following completion of 1D SDS-PAGE (Section 5.2.2), a western blot was performed; reagents and buffers are listed in Chapter 2, Table 2.2. The 1D SDS-PAGE gel was transferred from the plates into transfer buffer and the stacking gel was removed. The gel was then placed in a Mini Trans-Blot Module transfer cassette (Bio-Rad, Hemel Hempstead, UK) with a nitrocellulose membrane (Bio-Rad, Hemel Hempstead, UK) and placed in the electrophoresis tank and ran at 100 V, 240 mA for 1 hour and 20 minutes using a Bio-Rad powerpac 300 on a magnetic stirrer.

Once the antigens had transferred onto the nitrocellulose membrane, the membrane was removed from the cassette and washed in PBST for 5 minutes thrice. Overnight incubation at 4°C on a rocking platform with 5% (w/v) Marvel was carried out to block the membrane (to reduce subsequent non-specific protein binding). The membrane was then washed in PBST for 5 minutes on a rocking platform thrice and incubated with rabbit anti-treponemal polyclonal antibody (diluted 1/5000 with PBST) at room temperature for 1 hour on a rocking platform. The membrane was then washed in PBST for 5 minutes on a rocking platform thrice and incubated with the secondary antibody, goat anti-rabbit IgG conjugated to peroxidise (Sigma-Aldrich, Dorset, UK) (diluted 1/500 in PBST), at room temperature for 1 hour on a rocking platform. The membrane was then washed in PBST as previously described and incubated in 1 ml of TMB liquid substrate system for membranes (Sigma-Aldrich, Dorset, UK) at room temperature in darkness for 5-15 minutes for colour

development. The reaction was stopped with ddH_2O and the antibody bound to antigen could be visualised as a coloured product.

5.2.4 Immunohistochemistry and histopathology of PCR positive healthy tissues

Immunohistochemistry (IHC) was carried out by the Veterinary Pathology Services, School of Veterinary Science at the University of Liverpool (Leahurst Field Station, Wirral, UK). Analysis and photographs of labelled tissues was undertaken by Hayley Crosby-Durrani, who at the time of the study was a pathology resident at the School of Veterinary Science.

Gingiva, RAJ and healthy foot tissue (Chapter 4, Section 4.2.2) that was PCR positive for one or more of the three DD treponeme phylogroups (*T. medium*, *T. phagedenis* and *T. pedis*) were submitted for IHC and haematoxylin and eosin (HE) staining to investigate whether DD treponemes could be visualised in these tissues and whether they could be present without associated tissue damage. IHC was carried out with rabbit anti-treponemal polyclonal antibodies (Section 5.2.3) using an automated protocol optimised for the labelling of treponemes (Evans et al., 2009c; Crosby-Durrani et al., 2016).

Gingiva, RAJ and healthy foot tissues fixed in 10% Neutral buffered formalin (NBF; Sigma-Aldrich, Dorset, UK) (Section 4.2.2) were embedded in paraffin wax and sectioned using a microtome in to 4 µm sections. Sections were then either stained by HE using a standard protocol or underwent IHC. For IHC, sections were deparaffinised with a series of xylene and ethanol baths. Antigen retrieval was carried out with pH6.1 EnVision™ FLEX target retrieval solution (Dako, Agilent Technologies, Carpinteria, California, USA) at 95°C for 25 minutes. The following steps were performed on the sections using a DAKO Autostainer Link 48 (Dako, Agilent Technologies, Carpinteria, California, USA). Sections underwent a series of washes using EnVision™ FLEX wash buffer (Dako, Agilent Technologies, Carpinteria, California, USA). Sections were then subjected to EnVision™ FLEX peroxidise block (Dako, Agilent Technologies, Carpinteria, California, USA) to block endogenous peroxidise, thus preventing false positive labelling and then washed again. The sections were incubated for 20 minutes with primary antibody (rabbit anti-treponemal polyclonal antibodies) which had been diluted 1/4000 with EnVision™ FLEX antibody diluent (Dako, Agilent Technologies, Carpinteria, California, USA). Sections were washed and incubated with EnVison™ FLEX/HRP (Dako, Agilent Technologies, Carpinteria, California, USA), a labelled polymer, for 20 minutes followed by a series of washes. Bound antigen was

detected using EnVision™ FLEX DAB + chromogen (Dako, Agilent Technologies, Carpinteria, California, USA). Sections were counterstained with haematoxylin.

IHC was positively controlled for by including a grade 2 CODD coronary band to dorsal horn sample which had previously labelled positive for DD treponemes via IHC (Angell et al., 2015b). Several tissues that were PCR negative for the three DD treponeme phylogroups also underwent IHC as negative controls. The following grading system was used to describe the intensity of antigen presence for each sample: L0 = no labelling, L1 = mild granular labelling (interpreted as background) or L2 = intense labelling. Furthermore, it was also noted whether treponeme morphology could be observed.

5.2.5 Multi locus sequence typing of DD treponemes in host tissue samples

MLST carried out in this section was completed in partnership with Dr Simon Clegg, a Postdoctoral Research Associate at the University of Liverpool.

Multi locus sequence typing (MLST) of DD treponemes was carried out on either extracted genomic DNA from cultured gingiva (*n*=6), RAJ (*n*=1) and healthy foot tissue (*n*=14) samples containing DD treponemes but not sub-cultured on plates for isolation (not pure cultures) or directly from extracted genomic DNA from tissue samples that were PCR positive for DD treponemes in the gingiva (*n*=9) and RAJ (*n*=1), DD lesions (*n*=2) and healthy foot tissue (*n*=5) (Chapter 4 Sections 4.2.1-5). Extracted genomic DNA from a rumen reticular pillar, rumen dorsal sac, gingiva and lesion from a single dairy cow from a previous study were also kindly donated (Evans et al., 2012b). For some cultured healthy foot tissues, multiple DNA extracts of the same healthy foot culture have been analysed, which were taken at different points during the culture growth and maintenance. Overall, this resulted in 8 cows having extracted genomic DNA from more than one tissue location analysed by MLST and a further 17 cows with only one sample location analysed.

The extracted genomic DNA from culture underwent PCR assays specific for each of three DD treponeme phylogroups (see Chapter 2 Section 2.7) before preparation for MLST to determine which DD treponeme phylogroups were present. For some tissues and cultures, MLST was carried out for more than one DD treponeme phylogroup depending upon the DD treponeme specific phylogroup PCR assay results. The MLST protocol used in this study followed a protocol previously developed for a study investigating the population structure and diversity of DD treponemes isolated from cloven hoofed animals (Clegg et al., 2016b).

Samples were subjected to PCR assays specific for each of the three DD treponeme phylogroups using previously designed MLST primers which amplify a fragment (500-600bp) of each of the seven targeted genetic loci (Table 5.1). These genetic loci encode for adenosine kinase (ADK), glutamate dehydrogenase (GDH), glycerol kinase (GlpK), a heat shock protein (GroEL), orotidine 5'-phosphate decarboxylase (PyrG), recombination protein A (RecA) and the large RNA polymerase sub unit (RplB). PCR reaction mixes were set up as previously described with the DD treponeme phylogroup specific MLST primers (Chapter 2, Section 2.7.2). PCR cycling conditions for each assay was as follows: 95°C for 1 minute, followed by 40 cycles of 95°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, the final extension was carried out at 72°C for 10 minutes. PCR products were purified as previously described (Chapter 2, Section 2.7.1). Purified PCR products were sequenced commercially (Macrogen, Amsterdam, the Netherlands) and assembled as previously described (Chapter 2, Section 2.7.2).

The seven MLST loci sequence data for each phylogroup tissue culture or associated tissue were concatenated and aligned with concatenated sequences from other isolates including those from a previous DD treponeme MLST study (Clegg et al., 2016b). Phylogenetic inferences from this alignment of concatenated sequences were made. TN93 models were used to draw concatenated gene trees with 10 000 bootstrap values used to produce all maximum likelihood trees (Tamura and Nei, 1993). Screening of alignments for evidence of recombination was performed using SplitsTree4 (Huson and Bryant, 2005). GARD and SLAC, available through the Datamonkey web server, were used to screen alignments for positive and negative selection (Pond and Frost, 2005).

Table 5.1: Digital dermatitis treponeme multilocus sequence typing primers[a]

Locus	Putative gene protein	Position[1]	DD treponeme phylogroup	Predicted product size (bp)	Forward primer (5'-3')	Reverse primer (5'-3')
adK	Adenosine kinase	2265510-2265903	*T. medium*	517	CTGCAAAATATTATGGTATCCCTCA	GCATCCAAAGTTATGAGCAGTTTT
			T. phagedenis	499	GCTATCAAATCCCGCATATTTC	TTTGCGAGTACATTTTTCTTTTCAT
			T. pedis	526	TCAAAGTTGTACAAGATACCGCATA	ATGAGGGACGTGCGTCAATA
gdh	Glutamate dehydrogenase	275169-275682	*T. medium*	647	CGTCAATACTAACGGACAGATTATG	GGTTCTGTACCCATTCAAAGTAAGA
			T. phagedenis	643	GTCAACACAAACGGGCAAATAAT	TCTGAACCCATTCAAAGTAAGAAAC
			T. pedis	623	GTGGGTACAAATGCGAAAATTATG	CATTCAAAATACGAAACAATTACCC
glpK	Glycerol kinase	1797272-1797770	*T. medium*	613	TATTTTATCATTCGATCAGGGAACA	AATATTCAGTTCCGTCAGAATTTCA
			T. phagedenis	610	ATATTTTAGCACTTGATCAGGGAAC	CCGAGTTCTTGTAAAATCTCATCAT
			T. pedis	589	ATCTTTTGACCAAGGAACTACAAGT	TAACTCATTATCCCATTCCAAAGTC
groEL	Heat shock protein	768883-769428	*T. medium*	545	CTTGAATTAAAGCGCGGTATG	AAAATAGCGATATCTTCGAGCATT
			T. phagedenis	549	CTTGAGCTGAAACGAGGAATG	GGTAAGAATAGCAATATCTTCAAGCA
			T. pedis	542	GCTTGAATTAAAACGCGGAAT	CTGCAATATCTTCAAGCATTTCTTT
pyrG	Orotidine 5' phosphate dehydrogenase	2320945-2321441	*T. medium*	601	CAGGTTATCCCGCATGTTACC	ACGCTTCGCTTACGCTTAAATAC
			T. phagedenis	611	GTACAAGTTGTCCCGCATGTAAC	GCAGTCAGCGCTTCACTCAC
			T. pedis	596	GTACCCCATGTAACCGATGAA	AGGGCTTCCACTACGCTTAAATA
recA	Recombination protein A	2449887-2450338	*T. medium*	571	CTACAAATCGAAAAGGAGTTTGGA	CGTACGCAATACCGATTTTCAT
			T. phagedenis	572	GCCTTCAAATCGAAAAACAATTC	GAACATAACGCCGATTTTCAT
			T. pedis	560	AAATTGAAAAACAATTCGGACAG	AACACCGATTTTCATTCTTATTTGA
rplB	Large polymerase sub unit	953257-953715	*T. medium*	565	ATATAAGCCTATAACACCGGGTATG	ACCGATTGTTGCATAGCATTTT
			T. phagedenis	575	ATAAGCCTATAACACCGGGACTAAG	ATTTCCAACTTCACCGATTGTC
			T. pedis	575	TCTAAAAGAATATAAGCCGATGACG	CGCCTATGGTAGCATAACATTTTT

[a] *Table adapted from Clegg et al., (2016).*

[1] *Gene positions corresponding to T. vincentii OMZ 383 (Genbank accession code-CP009227).*

5.3 Results

5.3.1 Western blot to determine the specificity of the rabbit polyclonal anti-treponemal antibodies

Polyclonal anti-treponemal antibodies were previously raised against a cocktail of the three cultivable DD treponeme phylogroups (*T. medium*, *T. phagedenis* and *T. pedis*) in rabbits and successfully applied to immunohistochemistry (IHC) to visualise DD treponeme presence in DD lesional tissue. However, it is unknown whether these antibodies were specific to DD treponemes or if they experience some cross-reactivity with antigens from commensal DD treponemes colonising the GI tract. To determine whether the rabbit polyclonal anti-treponemal antibodies cross react with commensal GI treponemes, a western blot was carried out on antigen preparations from the GI treponemes, *T. ruminis* and *T. rectale* as well as *T. medium* DD treponeme phylogroup strain T19 for comparison. The results are shown in Figure 5.2.

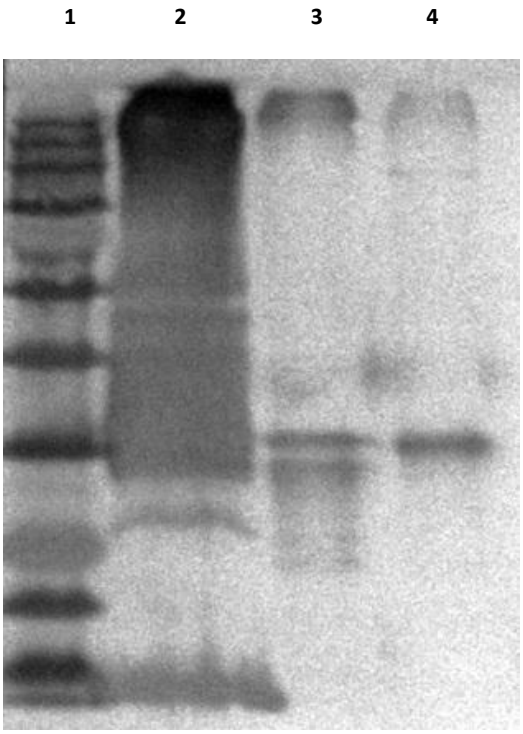


Figure 5.2 Western blot of rabbit anti-treponemal polyclonal antibody cross reactivity with commensal GI treponemes compared to T. medium DD treponeme phylogroup strain T19.

Lane 1 is the protein ladder 10-230 kDa, lane 2 is the T. medium DD treponeme phylogroup strain antigen preparation, lane 3 is the T. ruminis antigen preparation and *lane 4* is the *T. rectale antigen preparation.*

T. medium DD treponeme phylogroup strain T19 had a large amount of antigen to which the rabbit polyclonal anti-treponemal antibody bound to; leading to smeared staining with only a few distinct bands visible for the *T. medium* DD treponeme phylogroup strain T19 as shown in Figure 5.2. However, despite the lack of distinct bands for T19 what is clear from the western blot is that there is a small amount of cross reactivity of the rabbit polyclonal anti-treponemal antibodies with a few *T. ruminis* and *T. rectale* antigens. There were some similarities and variations in banding patterns for *T. ruminis* and *T. rectale* with a distinct protein band present at approximately 30 kDa for both bacteria, however, *T. rectale* has a distinct band present at approximately 10 kDa which was not present for *T. ruminis*. Furthermore, *T. ruminis* appears to have bands present between 25-30 kDa which are not visible for *T. rectale*. Due to the large amount of staining for *T. medium* DD treponeme phylogroup strain T19 it is largely not possible to compare banding patterns with *T. ruminis* and *T. rectale,* except for band staining at approximately 20 kDa for *T. ruminis* which is not present for T19. Whilst there is some cross reactivity of the rabbit polyclonal anti-treponemal antibody for commensal GI treponemes, *T. medium* DD treponeme phylogroup strain T19 and GI commensal treponemes have largely different antigens to one another as indicated by the large amount of staining for T19 and the low amount of staining for the commensal GI treponemes.

5.3.2 Understanding DD treponeme carriage in apparently healthy tissue via histopathology and immunohistochemistry

IHC and haematoxylin and eosin (HE) staining were carried out on a proportion of apparently healthy tissues that were positive by PCR for one or more of the DD treponeme phylogroups (*T. medium, T. phagedenis* and *T. pedis*) to determine if DD treponemes could be visualised in these tissues and whether there was any tissue abnormalities present microscopically that was not visible macroscopically (*n*=51). Furthermore, a small subset of apparently healthy tissues negative by PCR for DD treponemes also underwent IHC and HE staining for comparison with results from PCR positive samples (*n*=11). A summary of the most common findings can be found in Table 5.2 for each sample type. Figures 5.3-10 show examples of the histopathological and IHC findings for different types of samples.

5.3.2.1 Histopathology and immunohistochemistry of GI tract tissues

Of the eight gingiva samples positive by PCR for one or more of the three DD treponeme phylogroups (Chapter 4 Section 4.3.1.1), four samples underwent IHC with the rabbit

polyclonal anti-treponemal antibodies as well as HE staining and one sample underwent IHC only (total *n*=5). Problems cutting sections of tissue in the paraffin block for the other three gingiva samples meant staining could not be processed for these samples. All gingiva samples appeared as expected for normal gingival tissue with no abnormalities in appearance with the exception of a couple of samples exhibiting mild inflammation which is to be expected in the gingiva. Figure 5.3 a) and b) shows an example of one of these gingiva samples. All five samples did not have labelling consistent for the presence of treponemes (labelling grade L2) with the rabbit polyclonal anti-treponemal antibody, although three had background labelling (labelling grade L1). Gingiva samples (*n*=4) negative by PCR for the three DD treponeme phylogroups (two samples positive for the *Treponema* genus PCR) were also submitted for IHC and H&E staining and also showed no signs of abnormalities in the tissue associated with disease or labelling for treponemes, although some had background labelling (L1, *n*=3).

Only one RAJ sample was positive by PCR for DD treponemes (Chapter 4 Section 4.3.1.1) and analysis of the tissue by HE staining and IHC showed no abnormalities in the tissue indicative of disease or labelling for treponemes (Figure 5.4). The tissue was similar to the RAJ samples which were DD treponeme negative by PCR (one sample positive for the *Treponema* genus PCR) which also showed no abnormalities indicative of disease and no labelling for treponemes (Figure 5.5).

5.3.2.2 Histopathology and immunohistochemistry of pedal tissues

Healthy feet tissue samples were not as clear of infection as they appeared macroscopically. Of the 39 healthy feet samples positive by PCR for one or more of the DD treponeme phylogroups (Chapter 4 Section 4.3.1.2), 36 were submitted for IHC and 31 for HE staining as well. Table 5.2 contains a summary of the most common histopathological findings for the healthy foot samples examined. A proportion of the healthy foot samples (*n*=25) histologically showed similarities with the beginning of horn rather than skin, with severely thickened epidermis and the development of organised laminae-like structures. This may be a result of where the samples were taken from as shown in Chapter 4 Figure 4.1. However, extreme thickening of the epidermis as a result of DD lesion development can make distinguishing between the beginning of horn and foot skin samples difficult. For the purposes of this thesis, these samples will henceforth cautiously be named as suspected horn to differentiate them from definite skin samples.

Table 5.2: Summary of common histopathological findings from tissues which underwent HE staining and IHC

			Immunohistochemistry		Histopathology						
Sample type	DD treponeme PCR results	No. of samples examined[a]	Labelling Grades[b]	Treponeme morphology	Abnormalities	Thickening of epidermis[c]	Erosion of stratum corneum	Ulceration of epidermis	Necrotic material	Bacteria	Immune cell infiltration[d]
Gingiva	+	5	2 x L0, 3 x L1	-	-	-	-	-	-	-	-
RAJ	+	1	1 x L0	-	-	-	-	-	-	-	-
Healthy foot*	+	36 (5)	2 x L0, 7 x L1, 27 x L2	16	28	16	13	5	7	21	16
A) Suspected horn	*+*	*25 (4)*	*1 x L0, 2 x L1, 22 x L2*	*15*	*22*	*11*	*12*	*5*	*6*	*17*	*11*
B) Haired skin	*+*	*10*	*1 x L0, 4 x L1, 5 x L2*	*1*	*6*	*5*	*1*	*0*	*1*	*4*	*5*
C) Coronary band	*+*	*1 (1)*	*1 x L1*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
Other	1+/8-	9	1 x L0, 6 x L1, 2 x L2	1	6	6	1	0	1	1	1
Healthy gingiva controls	-	4	1 x L0, 3 x L1	-	-	-	-	-	-	-	-
Healthy RAJ controls	-	3	2 x L0, 1 x L1	-	-	-	-	-	-	-	-
Healthy foot controls	-	4	4 x L0	-	-	-	-	-	-	-	-
Total		62	29	17	34	24	14	5	9	21	18

Results indicate number of tissues which were positive for a particular characteristic.

[a] *( ) contain the number of samples that only underwent IHC.*

[b] *Labelling of tissues for treponemes was carried out using rabbit polyclonal anti-treponemal antibodies raised against the three cultivable DD treponemes: T. pedis, T. phagedenis and T. pedis. L0 = no labelling, L1 = mild granular labelling (interpreted as background) or L2 = intense labelling.*

[c] *Includes thickening of the stratum corneum by both parakeratotic hyperkeratosis and orthokeratotic hyperkeratosis.*

[d] *Refers to samples which displayed moderate to severe or multifocal perivascular infiltrates of lymphocytes, plasma cells and/or neutrophils.*

** Healthy foot total results. Below, in italics, the total healthy foot results are subdivided based on foot sample type: A) suspected horn, B) haired skin and C) coronary band.*

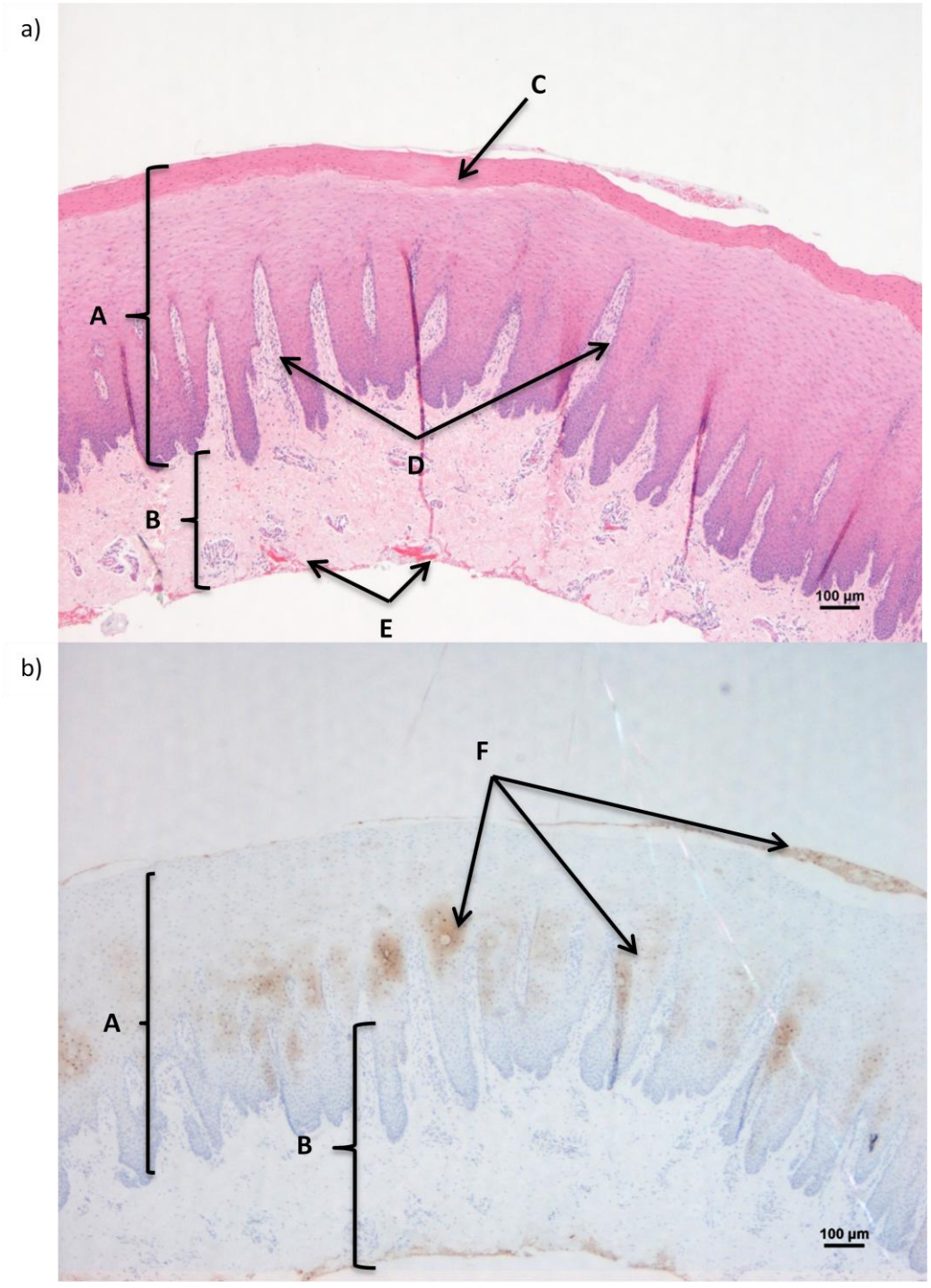


Figure 5.3: DD treponeme PCR positive gingiva sample histopathology and IHC sections

Sample 318. a) Cross section of gingival tissue sample, positive by PCR for the T. medium DD treponeme phylogroup, stained with HE encompassing the mucosa (A) with keratinised stratified squamous epithelium (C) and the lamina propria (B). A small number of scatter plasma cells and lymphocytes (D) are present at the interface of the mucosa and lamina propria. Extravasated erythrocytes (E) are present at the periphery and are likely a sampling artefact. b) The same cross section of gingiva following IHC with rabbit polyclonal anti-treponemal antibodies (brown labelling, F). Labelling grade L1 (background labelling). Magnification x4 for both figures a) and b).

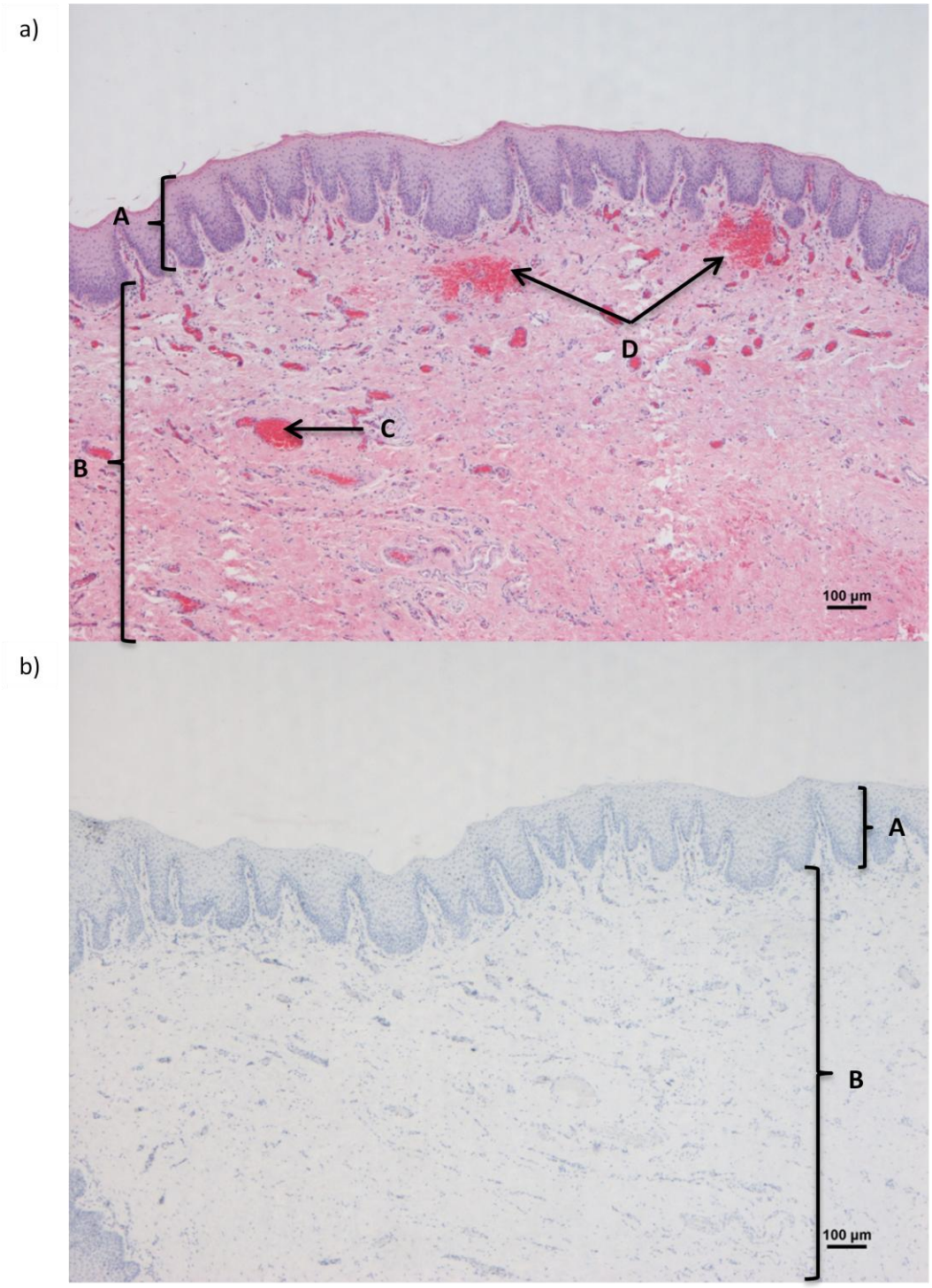


Figure 5.4: DD treponeme PCR positive RAJ sample histopathology and IHC

Sample 384. a) HE staining of a section of RAJ sample, positive by PCR for the T. phagedenis DD treponeme phylogroup, encompassing the mucosa (A) and submucosa (B). Hyperaemic blood vessels are present in the submucosa (C) a long with multifocal extravasated erythrocytes (D) (sampling artefact). No abnormalities present indicative of disease. b) IHC labelling of sample with rabbit polyclonal anti-treponemal antibodies. No labelling (labelling grade L0) present as indicated by the lack of brown colour on the section. Magnification x4 for both figures a) and b).

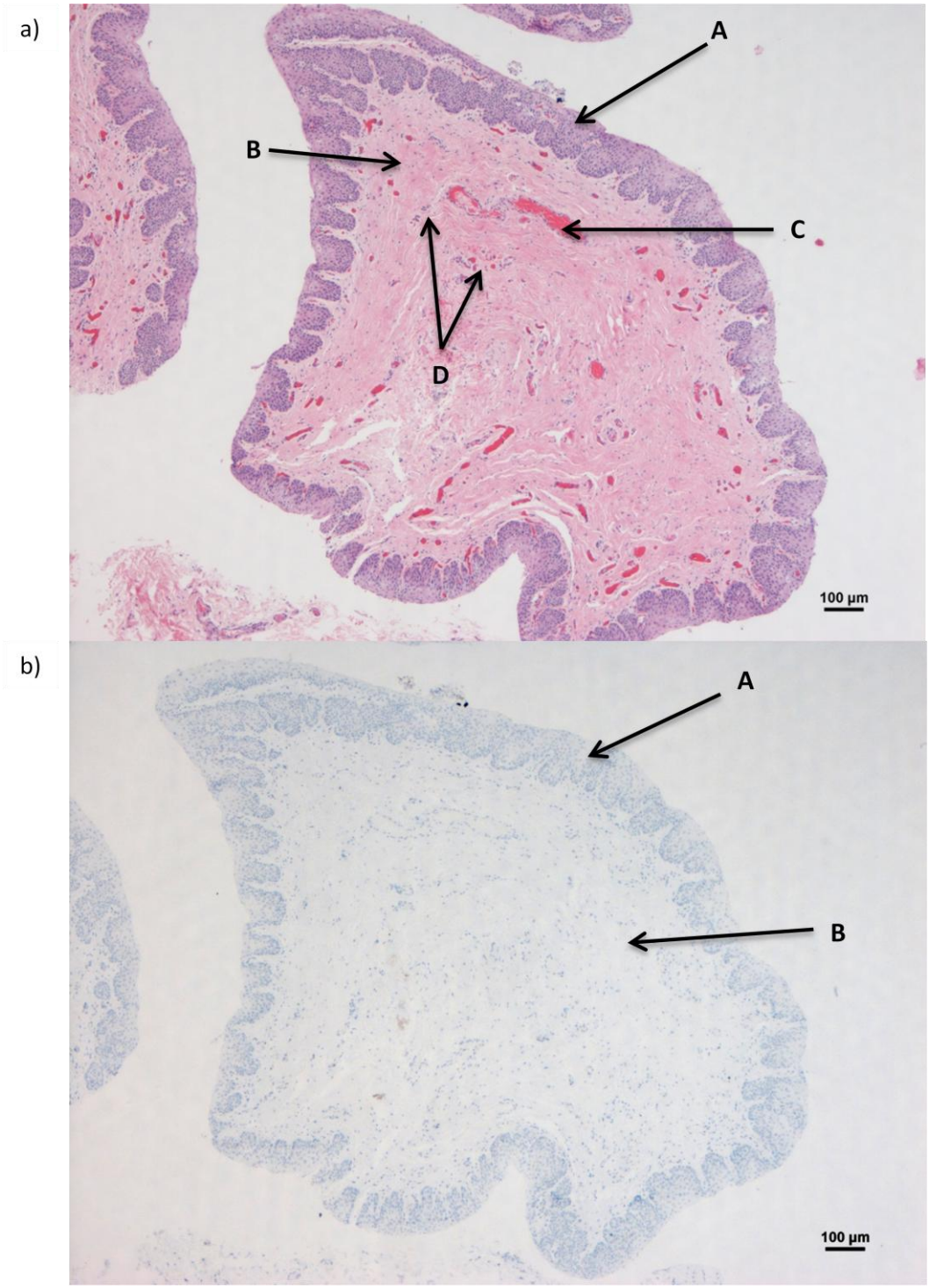


Figure 5.5: DD treponeme PCR negative RAJ histopathology and IHC

Sample 213. a) HE staining of a RAJ sample, negative by PCR for the T. medium, T. phagedenis and T. pedis DD treponeme phylogroups, encompassing the mucosa (A) and submucosa (B). In the submucosa, hyperaemic blood vessels are present (C) as well as mild multifocal perivascular infiltrates of lymphocytes and plasma cells (D). No abnormalities present indicative of disease. b) IHC treatment of the sample for DD treponemes with rabbit polyclonal anti-treponemal antibodies resulted in no labelling (labelling grade 0). Magnification x4 for both figures a) and b).

Abnormalities not normally seen in healthy horn were observed in 22 suspected horn samples. In some samples thickening of the epidermis (*n*=11) was exhibited either through parakeratotic hyperkeratosis (*n*=6) or more commonly orthokeratotic hyperkeratosis (*n*=11) (Figure 5.6 and 5.9). Erosion of the stratum corneum occurred in 12 of the healthy suspected horn samples (Figure 5.7a) whereas changes consistent with ulceration of the epidermis were present in five of the samples (Figure 5.8). Many samples (*n*=11) had multifocal, moderate to severe perivascular infiltrates of lymphocytes and plasma cells in the dermis with varying levels of neutrophils present which were often degenerate (Figure 5.8 and 5.9). Necrotic tissue was observed in six samples. Bacteria could be visualised in a large proportion of samples (*n*=17) (Figure 5.6, 5.8 and 5.9), usually embedded in the stratum corneum and often associated with the presence of plant material also embedded in the stratum corneum (Figure 5.6). A couple of samples also showed bacteria extending down into the epidermal fissures (Figure 5.9). Other abnormal observations seen in a small number of suspected horn samples included the presence of a serocellular crust, plump activated endothelial cells in capillaries, mild perivascular lymphoplasmacytic dermatitis and extravasated erythrocytes (haemorrhage) (Figure 5.9).

With regards to the IHC results of the healthy suspected horn samples, labelling with the rabbit polyclonal anti-treponemal antibody, indicating treponeme presence (labelling grade L2), occurred in 22 samples with treponeme morphology observed in 15 of these samples. Typically intense extracellular grade L2 labelling (which is sometimes granular) was observed on the very surface of the epidermis and sometimes between the layers of the stratum corneum (Figure 5.7b and 5.9c), with the most intense labelling in the upper most superficial layers. There was one sample in which the labelling followed the epidermal laminae down and another in which mild labelling tracked down into the epidermal fissures from the surface (Figure 5.9c). If treponeme morphology was observed, it was around the periphery of areas of intense labelling (Figure 5.7c).

Haired skin accounted for 10 of the healthy skin samples. Like the suspected horn samples, six haired skin samples had changes which were not considered normal for this sample. Overall a total of five samples exhibited a thickening of the epidermis. Thickening of the stratum corneum by parakeratotic hyperkeratosis was observed in four of these samples and thickening as a result of orthokeratotic hyperkeratosis was observed in all five samples. A single sample exhibited erosion of the stratum corneum and necrotic material could also be visualised in a single sample. Half of the samples (*n*=5) had perivascular infiltrates of

lymphocytes and plasma cells and/ or degenerate neutrophils in the superficial dermis which may also be infiltrating into the stratum corneum. In four samples, bacteria could be observed; again these bacteria were often associated with samples that had plant material embedded in the stratum corneum. Severely hyperaemic capillaries in the superficial dermis were also observed in a sample.

Labelling of the haired skin with the rabbit polyclonal anti-treponemal antibodies indicating the presence of treponemes (labelling grade L2) occurred in half of the samples, with treponeme morphology observed in only one sample. Extracellular labelling (sometimes granular) was predominantly at the surface of the epidermis following the outline of the keratinocytes, although in one sample labelling was focused around necrotic material and in another labelling tracked down into the fissures from the surface. Again, like the horn, if treponeme morphology was observed it was around the periphery of intense labelling.

A single sample of healthy foot tissue was classified as coronary band. This sample was not considered to have any abnormalities indicative of disease and only background labelling (grade L1) was observed by IHC.

Interestingly for healthy foot tissue, labelling of treponemes present in the sample via IHC did not always coincide with abnormalities present in the tissue. Indeed, two samples which labelled positively for treponemes showed no signs of abnormalities such as thickening of the epidermis associated with disease/ infection. Another sample, which was only submitted for IHC, was also positively labelled for treponemes and the IHC slide showed no obvious signs of abnormalities. However, as it was not HE stained, more subtle abnormalities could have been missed. The reverse was also true for healthy feet where abnormalities in the tissue were observed without labelling of treponemes in four samples. Again one of these samples had no HE staining carried out on the sample. However, ulceration of the tissue was clear on the IHC section analysed.

To act as controls, four samples of healthy foot (two horns, one haired skin and one unclassified) were submitted for HE staining and IHC which were either negative by PCR for both DD treponemes and the *Treponema* genus or positive for the *Treponema* genus only. The samples showed no abnormalities and no labelling for treponemes.

A proportion of foot samples (*n*=9) that could not be classified as lesions or healthy skin (see Chapter 4 Section 4.2.2) were also submitted for HE staining and IHC to try and gain further insights into these samples. Only one of these samples was positive by PCR for DD

treponemes and another five were positive for the *Treponema* genus only (Chapter 4 Section 4.3.1.2). A total of five of the samples were classified as horn, two samples as haired skin and another two samples as coronary band. Abnormalities were observed in six of the samples, spanning all foot sample type classifications and included the sample positive by PCR for DD treponemes. Two samples showed thickening of the stratum corneum by parakeratotic hyperkeratosis as well as orthokeratotic hyperkeratosis and the other four samples showed thickening by orthokeratotic hyperkeratosis only. Erosion of the epidermis was only observed in one sample. Bacteria and plant material embedded in the severely thickened epidermis was observed in one sample and this sample also had hyperaemic dermal laminae. In the sample that was PCR positive for DD treponemes, as well as a thickened epidermis, there were also intracorneal pustules (Figure 5.10) composed of necrotic material, degenerate neutrophils with extravasated erythrocytes (haemorrhage) present.

Intense labelling of treponemes (labelling grade L2) with the rabbit polyclonal anti-treponemal antibody was present in two samples which also had abnormalities present in the tissue, one of which was the sample positive for DD treponemes by PCR and the other was positive for the *Treponema* genus only. Both samples had labelling (sometimes granular) in the epidermis tracking down into the fissures however, treponeme morphology was only observed in the latter sample of the two samples. The latter sample was the sample described above with bacteria and plant material embedded in the thickened epidermis.

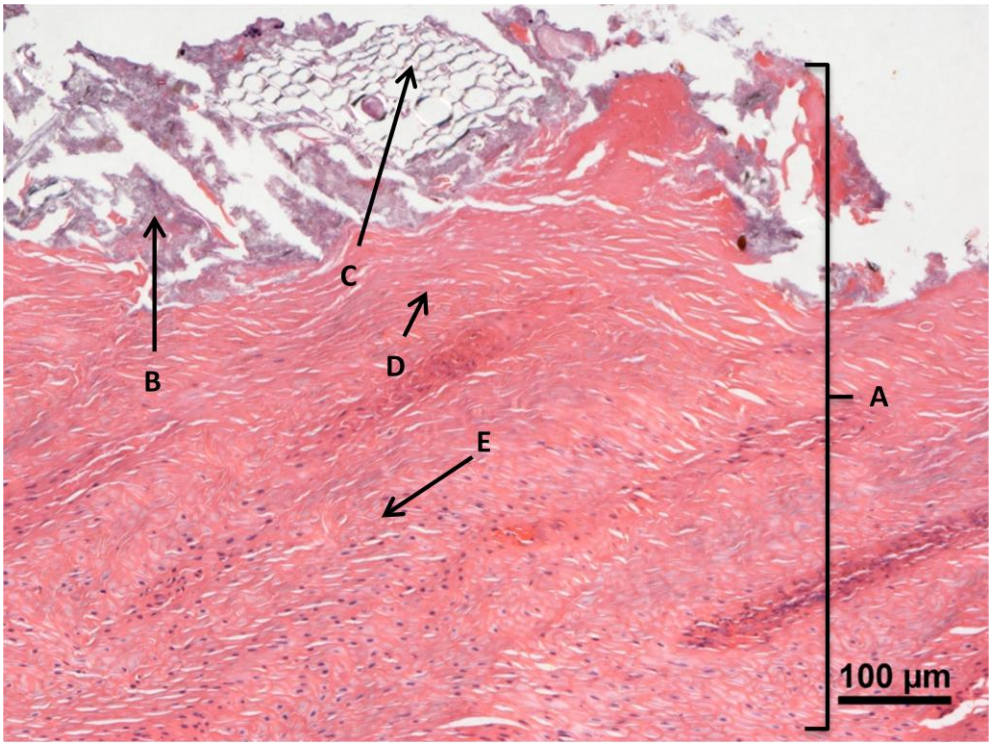
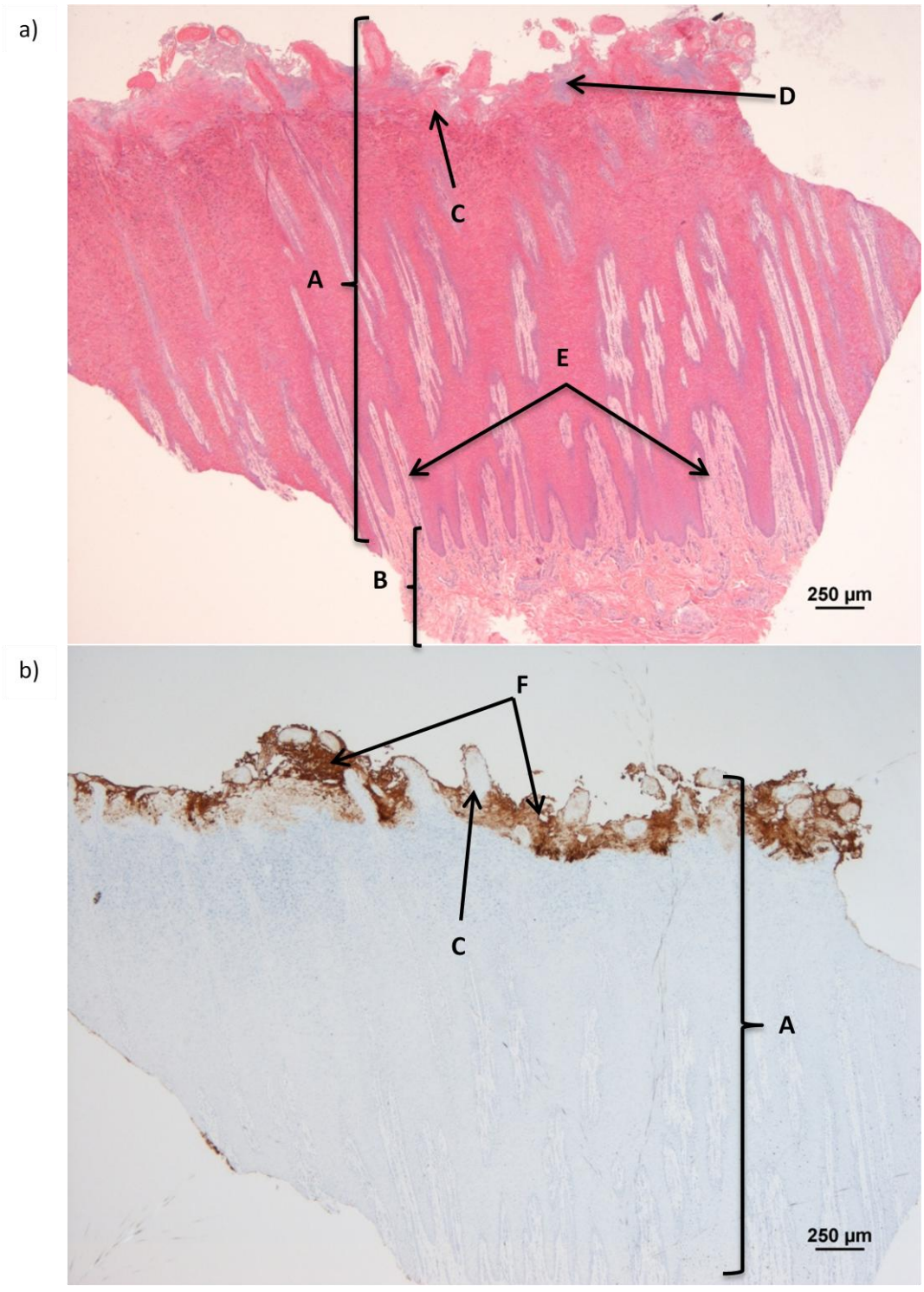


Figure 5.6: Thickened stratum corneum if a DD treponeme positive healthy foot suspected horn sample

Sample 532. HE staining of a healthy foot suspected horn sample positive by PCR for the T. medium and T. phagedenis DD treponeme phylogroups. Bacteria (B) and plant cells (C) are embedded in the irregular surface of the stratum corneum (A) which has been irregularly thickened by orthokeratotic (D) and parakeratotic (E) hyperkeratosis. Magnification x10.


a)
A
B
C
D
E
250 µm
b)
C
F
A
250 µm

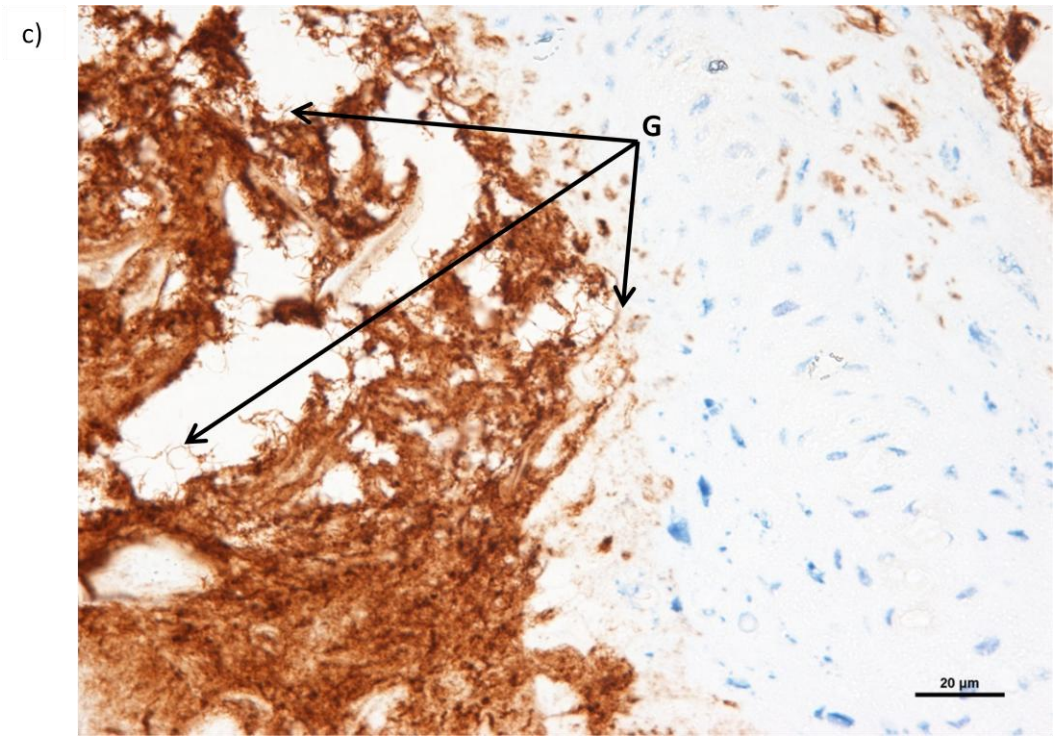


Figure 5.7: DD treponeme PCR positive healthy foot suspected horn sample histopathology and IHC

Sample 393. a) HE stained section of healthy suspected horn, positive by PCR for the T. medium, T. phagedenis and T. pedis DD treponeme phylogroups, encompassing the epidermis (A) and dermis (B) The stratum corneum is severely eroded (C) and bacteria (D) are embedded in the irregular surface. Throughout the dermal laminae there are mild infiltrates of lymphocytes and plasma cells (E). Magnification x2. b) IHC of a section of the same sample encompassing epidermis (A) using rabbit polyclonal anti-treponemal antibodies. Intense labelling (brown colour, labelling grade L2) for DD treponemes (F) present along the surface of the eroded stratum corneum (C). Magnification x2. c) Increased magnification (x40) of the above IHC section showing L2 grade labelling with treponeme morphology (G) at the periphery of labelling.

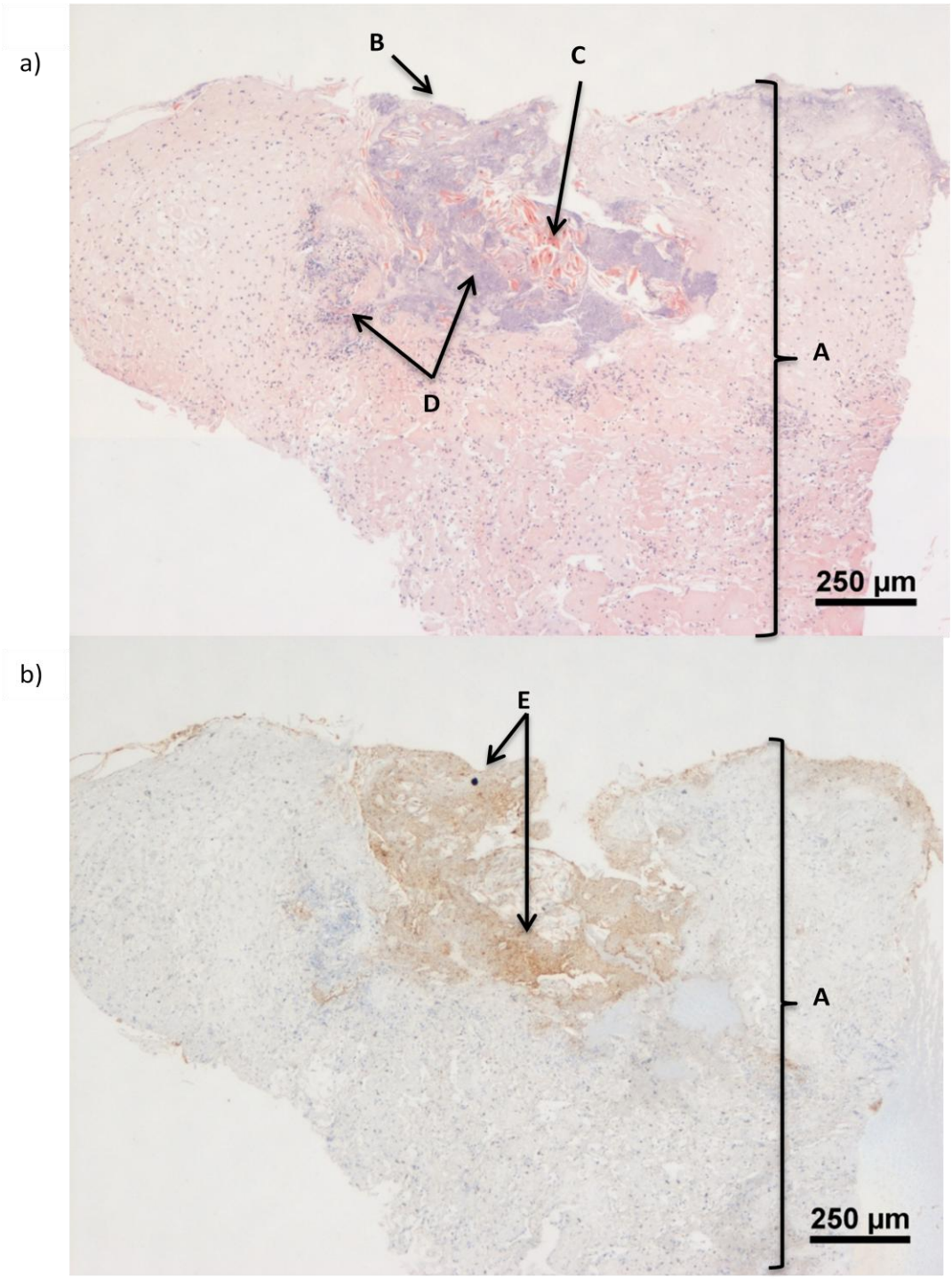
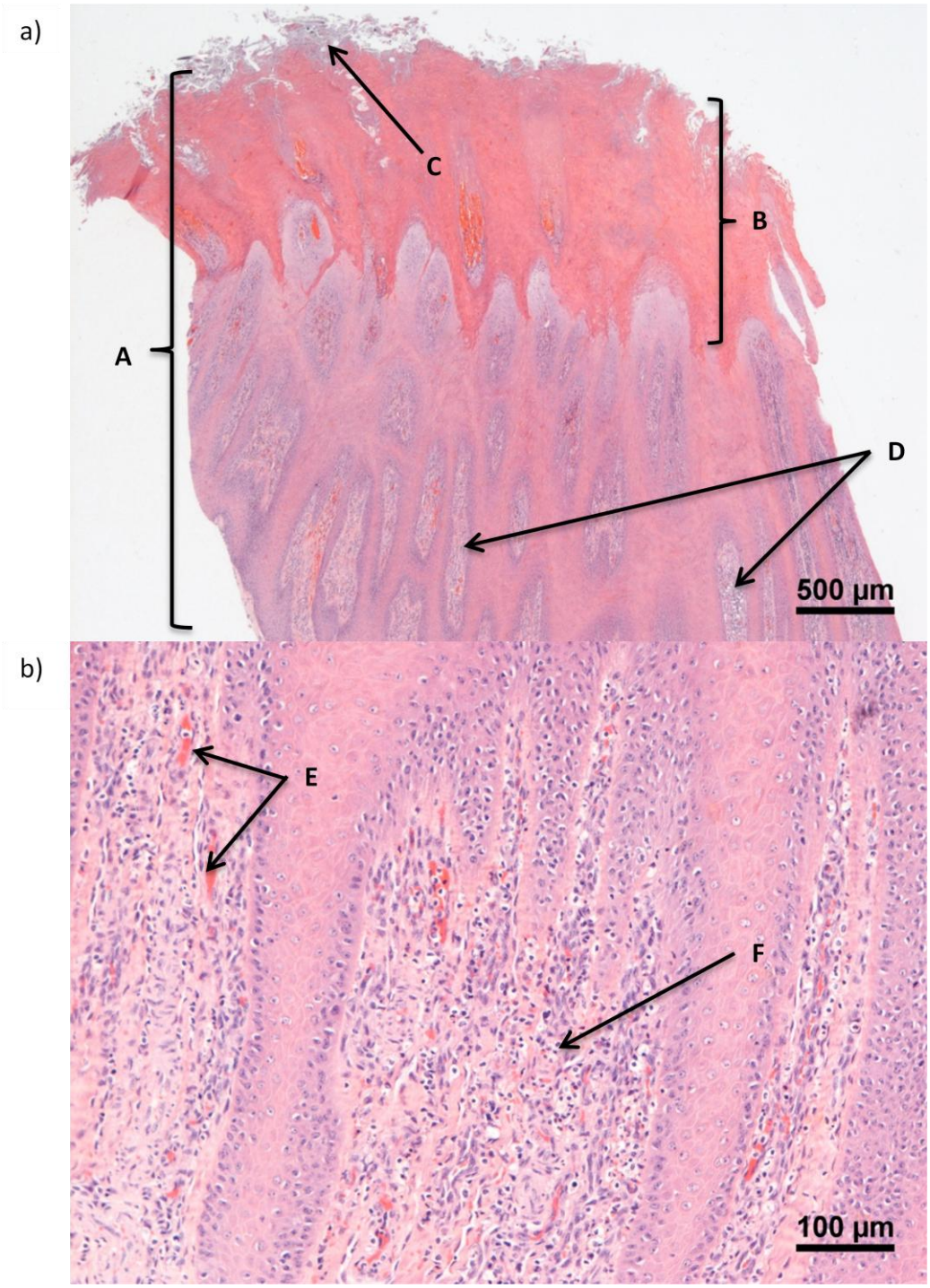


Figure 5.8: Ulceration of a healthy foot suspected horn sample positive by PCR for DD treponemes

Sample 252. a) HE stained section of a healthy suspected horn, positive by PCR for the T. pedis DD treponeme phylogroup, encompassing only the dermis (A) due to ulceration of the tissue resulting in complete loss of the epidermis (B). Necrotic keratinocytes (C) are surrounded by a mass of bacteria (dark purple staining) admixed with degenerate neutrophils which also extend out and around the periphery of the bacterial mass (D). b) IHC of a section of the same sample encompassing the dermis (A) using rabbit polyclonal anti-treponemal antibodies for DD treponeme detection. Mild background labelling (grade L1) present in the areas where bacteria are concentrated as well as surface of the dermis. Magnification x4 for both figures a) and b).


a)
C
B
A
D
500 µm
b)
E
F
100 µm

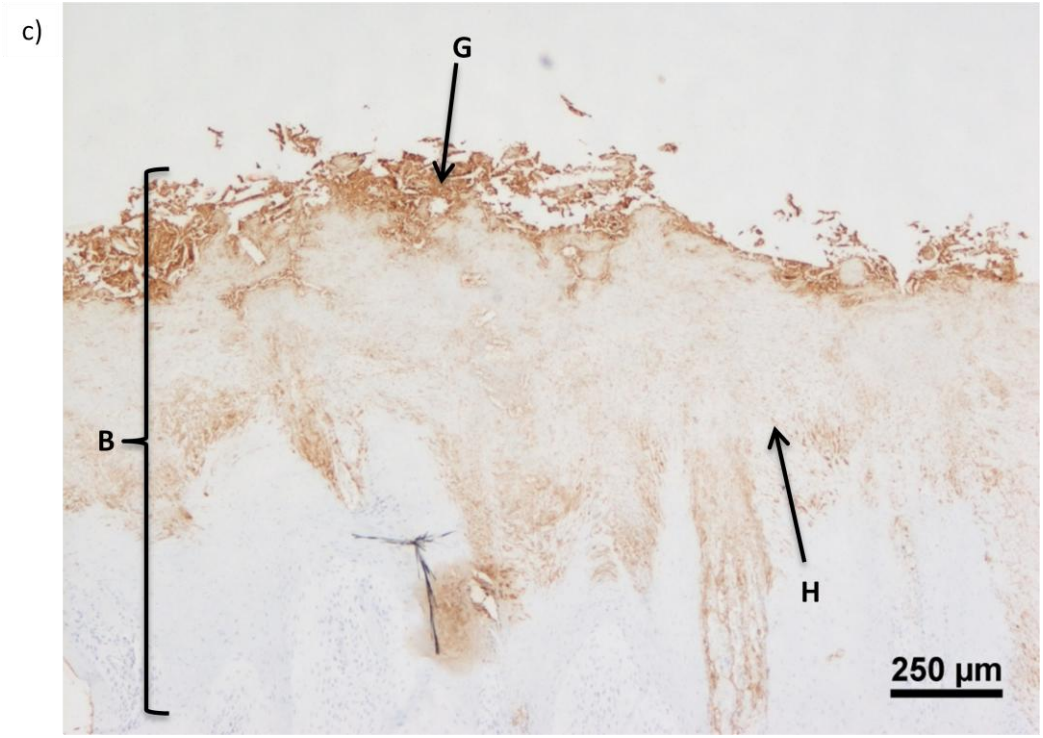


Figure 5.9: DD treponeme PCR positive healthy foot suspected horn sample with inflammation in the dermal laminae

Sample 483. a) HE stained section of suspected hoof horn, positive by PCR for the T. phagedenis DD treponeme phylogroup, encompassing the epidermis (A). The stratum corneum (B) is thickened by orthokeratotic and parakeratotic hyperkeratosis with bacteria (C) extending down from the surface of the stratum corneum into the fissures. Within the dermal laminae (D) there are moderate to severe infiltrates of inflammatory cells. Magnification x2. b) Magnified (x10) view of the dermal lamine from figure a) which contain extravasated erythrocytes (E, dark pink staining) and infiltrates of inflammatory cells (F) comprised of degenerate neutrophils lymphocytes, plasma cells and macrophages which are admixed with necrotic debris. c) IHC section encompassing the stratum corneum (B) of the same sample labelled with rabbit polyclonal anti-treponemal antibodies. Intense labelling (grade L2) of DD treponemes is observed at the surface of the stratum corneum (G) with a large area of background labelling (grade L1) extending further into the stratum corneum and into the fissures where in places labelling becomes slightly more intense again. Magnification x4.

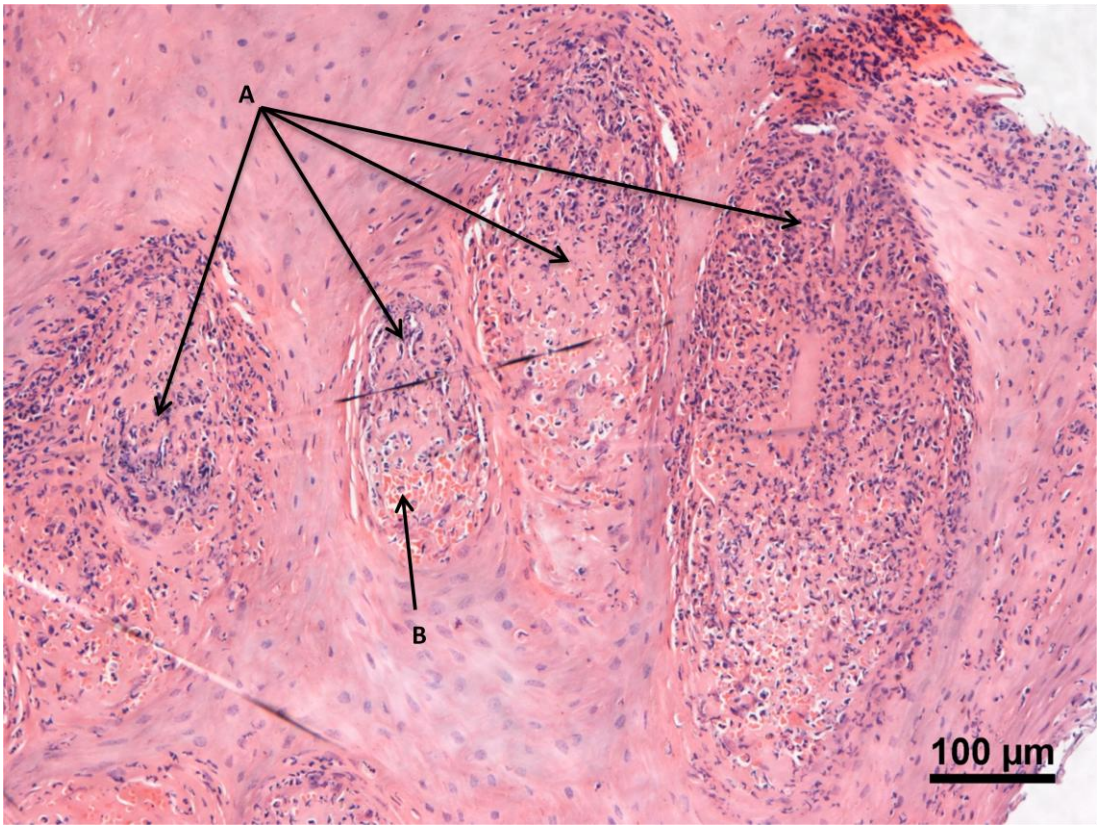


Figure 5.10: Intracorneal pustules present in a suspected horn sample from a foot classified as 'other' which was positive by PCR for DD treponemes

Sample 637. HE stained section of the severely thickened epidermis of an 'other' foot suspected horn sample which was positive by PCR for the T. phagedenis DD treponeme phylogroup. Within the epidermis there are multifocal intracorneal pustules (A) composed of degenerate neutrophils, necrotic debris and extravasated erythrocytes (B, arrow points to extravasated erythrocytes). Magnification x10.

5.3.3 MLST of tissues and cultures positive for DD treponemes

In order to carry out a relevant MLST approach both from culture and direct from tissue, those tissues identified as containing DD treponeme DNA using PCR were firstly reviewed and chosen as described below (5.3.3.1). Subsequently (5.3.3.2) each tissue culture and/or tissue were subjected to the MLST specific for the respective phylogroups to further determine the molecular epidemiology of DD treponemes present in different tissue types compared to strains present in DD lesions from different species.

5.3.3.1 DD treponeme phylogroup specific PCR assays

DD treponeme phylogroup specific PCR assay results for the extracted genomic DNA direct from tissue (Chapter 4, Section 4.3.1) as well as from culture were used to determine which

DD treponeme phylogroups should be targeted by MLST per sample. For gingiva tissue, three tissues were positive for the *T. medium* phylogroup, five tissues were positive for the *T. phagedenis* phylogroup and three tissues were positive for the *T. pedis* phylogroup. With regards to gingiva cultures, one gingiva culture was positive for the *T. medium* phylogroup, six gingiva cultures were positive for the *T. phagedenis* phylogroup and three gingiva cultures were positive for the *T. pedis* phylogroup. The *T. phagedenis* DD treponeme phylogroup was detected in each of the RAJ tissue and RAJ culture as well as the three DD lesional tissues examined. Of the healthy feet samples, three tissues and four cultures were positive for the *T. medium* DD treponeme phylogroup, five tissues and 14 cultures were positive for the *T. phagedenis* DD treponeme phylogroup and three tissue and five cultures were positive for the *T. pedis* DD treponeme phylogroup. The reticular pillar and rumen dorsal sac were both positive for the *T. phagedenis* DD treponeme phylogroup only. This resulted in a total of 28 tissues and 32 cultures that were subjected to MLST.

5.3.3.2 MLST results

For the benefit of this chapter section 'isolate data' refers to MLST data previously published by Clegg et al., (2016b) for isolates from DD lesions of various species (dairy and beef cattle, sheep, goats, elk and pigs) as well as the nearest human equivalents and 'isolate / tissue combined data' refers to 'isolate data' combined with the tissue and tissue culture MLST data generated in this chapter.

Sequences from the seven MLST loci were obtained for all 28 tissues and 32 tissue cultures which were positive for DD treponemes by PCR. Within each of the DD treponeme phylogroups there was variation at each of the loci for the isolate / tissue combined data (Table 5.3). *T. vincentii* was excluded from the comparative analysis as it was previously shown to be distinct from the *T. medium* DD treponeme phylogroup (Clegg et al., 2016b). For the isolate/ tissue combined data, the largest loci sequence variation range observed between the three DD treponeme phylogroups was for the *adk* locus in which the variability ranged from 0.5% in the *T. phagedenis* DD treponeme phylogroup to 7.0% in the *T. medium* DD treponeme phylogroup. The *T. medium* DD treponeme phylogroup demonstrated the highest variability within its loci than the other two DD treponeme phylogroups, with loci variation ranging from 2.1% at *gdh* to 7.0% at *adk* (*T. medium* phylogroup mean variation = 4%). Although the range of variability was slightly higher for the *T. pedis* DD treponeme phylogroup where the range was between 1.0% for *glpK* and 6.7% for *pyrG* (mean =3.1%). Whereas for the *T. phagedenis* DD treponeme phylogroup,

overall variation of loci was more conserved between 0.5% for *adk* and 1.8% for *gdh* (mean = 1.2%).

Table 5.3: MLST - Summary of individual loci analysis for 'isolate/ tissue combined data' compared to 'isolate data' [a]

Phylogroup	Locus	Amplicon size (bp)	No. (%) of variable sites DNA[b] [Isolate data]	No. (%) of variable sites aa[b] [Isolate data]	No. of DNA alleles [Isolate data]	No. of aa alleles [Isolate data]
***T. medium* (*n*=44)** [*n*=33]	*groEL*	448	**15 (3.3)** [15 (3.3)]	**0 (0.0)** [0 (0.0)]	**3** [3]	**1** [1]
	recA	475	**12 (2.5)** [12 (2.5)]	**11 (6.9)** [11 (6.9)]	**3** [3]	**3** [3]
	glpK	507	**22 (4.3)** [20 (3.9)]	**10 (5.9)** [18 (10.7)]	**4** [4]	**4** [4]
	adk	416	**29 (7.0)** [27 (6.5)]	**23 (16.6)** [23 (16.6)]	**5** [5]	**5** [5]
	gdh	514	**11 (2.1**) [11 (2.1)]	**2 (1.2)** [2 (1.2)]	**4** [4]	**4** [4]
	pyrG	501	**22 (4.4)** [21 (4.4)]	**18 (10.8)** [18 (10.8)]	**6** [4]	**5** [4]
	rplB	469	**11 (2.3)** [8 (1.7)]	**8 (5.1)** [8 (5.1)]	**4** [4]	**4** [4]
***T. phagedenis* (*n*=109)** [*n*=71]	*groEL*	456	**6 (1.3)** [6 (1.3)]	**4 (2.6)** [4 (2.6)]	**3** [3]	**2** [2]
	recA	472	**12 (2.5)** [12 (2.5)]	**4 (2.5)** [4 (2.5)]	**9** [9]	**4** [4]
	glpK	521	**4 (0.7)** [4 (0.7)]	**3 (1.7)** [3 (1.7)]	**5** [5]	**4** [4]
	adk	394	**2 (0.5)** [2 (0.5)]	**1 (0.8)** [1 (0.8)]	**3** [3]	**2** [2]
	gdh	560	**10 (1.8)** [10 (1.8)]	**9 (4.8)** [9 (4.8)]	**6** [5]	**4** [3]
	pyrG	527	**5 (0.9)** [5 (0.9)]	**1 (0.6)** [0 (0.0)]	**3** [3]	**2** [2]
	rplB	475	**3 (0.6)** [3 (0.6)]	**2 (1.3)** [2 (1.3)]	**3** [3]	**2** [2]
***T. pedis* (*n*=28)** [*n*=17]	*groEL*	441	**13 (2.9)** [13 (2.9)]	**0 (0.0)** [0 (0.0)]	**4** [4]	**1** [1]
	recA	477	**10 (2.1)** [10 (2.1)]	**2 (1.3)** [0 (0.0)]	**6** [6]	**3** [3]
	glpK	508	**5 (1.0)** [5 (1.0)]	**5 (3.0)** [5 (3.0)]	**4** [4]	**4** [4]
	adk	421	**13 (3.1)** [13 (3.1)]	**3 (2)** [3 (2)]	**5** [5]	**2** [2]
	gdh	520	**22 (4.2)** [22 (4.2)]	**16 (9.2)** [16 (9.2)]	**5** [5]	**5** [5]
	pyrG	507	**34 (6.7)** [21 (4.1)]	**1 (0.6)** [0 (0.0)]	**6** [5]	**2** [1]
	rplB	502	**10 (2.0)** [10 (2.0)]	**0 (0.0)** [0 (0.0)]	**4** [4]	**1** [1]

[a] *Analysis data is of tissues and tissue cultures from this study combined with previous data from DD treponeme isolates from various species (dairy and beef cattle, sheep, goats, elk, pigs and humans) (isolate/ tissue combined data)* (Clegg et al., 2016b) *is detailed in bold. [ ] contains data from previous isolates from various species DD lesions and human equivalent isolates only (isolate data)* (Clegg et al., 2016b)

[b] *T. vincentii was excluded from T. medium phylogroup analysis as it appears distinct from this phylogroup.*

When isolate/ tissue combined data was compared to the isolate data for loci variability (Table 5.3), there appeared to be more sequence variation for the isolate/ tissue combined data at the *T. medium* phylogroup *glpK, adk, pyrG* and *rlpB* loci than for the isolate data.

Additionally there was also more variation at the *T. pedis* phylogroup *pyrG* locus for the isolate/ tissue combined data than for the isolate data. There was no difference in variation for the *T. phagedenis* phylogroup loci between the two data sets.

The number of DNA alleles for loci per DD treponeme phylogroup for the isolate/ tissue combined data ranged from three to six, with the presence of novel alleles not previously described (Clegg et al., 2016b). The loci with the largest number of DNA alleles (*n*=6) in the *T. pedis* phylogroup were *pyrG* and *recA*, similarly *pyrG* had the largest number of alleles (*n*=6) for *T. medium* phylogroup and the *recA* loci had the largest number (*n=9*) for the *T. phagedenis* phylogroup (Table 5.3). When compared to the isolate data the *T. medium* phylogroup had two more DNA alleles at the *pyrG locus*. For the *T. pedis* phylogroup loci there was one more DNA allele at the *pyrG* locus. With regards to the *T. phagedenis* DD treponeme phylogroup, there was an additional allele at the *gdh* locus when compared to the isolate data.

Each allele for each loci was assigned a number unique to that allele (Table 5.4-5.6) with reference to the alleles discovered for the same MLST loci from DD lesion isolates previously analysed by MLST ('isolate data') (Clegg et al., 2016b). Alleles that were the same as those previously analysed with the isolate data were designated the same number. New alleles not seen before were given the next unassigned consecutive number.

MLST allelic profiles were then used to assign a sequence type (ST) to the tissues and tissue cultures. There was a total of four STs (ST1, 4, 12 and 13) identified for the *T. medium* DD treponeme phylogroup with two new STs (ST12 and 13). ST12 contained a novel allele at the *pyrG* locus whereas the rest of the allelic profile remained the same as ST1 (Table 5.4). ST13 also contained another new allele at the *pyrG* locus and the rest of the loci for that ST bare similarity to the ST4 allelic profile. For the *T. phagedenis* DD treponeme phylogroup there was 16 different STs assigned (ST1, 2, 3, 17, 20, 21, 22, 27, 36, 37, 38, 39, 40, 41, 42 and 43) and eight of these STs (ST 36-43) were not previously seen before (Table 5.5). New allelic profiles of known alleles accounted for seven of these new STs whereas ST41 was due to a new allele at the *gdh* locus. There were ten ST types identified for the *T. pedis* DD treponeme phylogroup (Table 5.6) and interestingly they were all novel STs. The majority of the novel STs were due to new allelic profile combinations of already reported alleles, however, two novel STs (ST10 and 16) contained a new allele (allele 6) at the *pyrG* locus.

Table 5.4: T. medium DD treponeme phylogroup sample ST summary

Sample ID[a]	Tissue type	MLST allele							ST
		adk	*gdh*	*glpK*	*groEL*	*pyrG*	*recA*	*rplB*	
318T	Gingiva	1	1	1	1	1	1	1	**1**
496T	Gingiva	1	1	1	1	1	1	1	**1**
609T	Gingiva	1	1	1	1	1	1	1	**1**
321C	Foot	1	1	1	1	1	1	1	**1**
253C	Foot	1	1	1	1	1	1	1	**1**
645C	Gingiva	1	1	1	1	1	1	1	**1**
568C	Foot	1	1	1	1	1	1	1	**1**
587C	Foot	1	1	1	1	1	1	1	**1**
494T	Foot	3	1	1	1	1	1	1	**4**
490T	Foot	1	1	1	1	6	1	1	**12**
495T	Foot	3	1	1	1	5	1	1	**13**

[a] *T denotes DNA extract from tissue sample and C denotes DNA extract from culture sample*

Only 2/11 of the tissue and tissue cultures combined had the same ST (ST13) for the *T. pedis* phylogroup, all other ST types were found in a single sample only. On the other hand, of the four STs identified for the *T. medium* phylogroup, 8/11 (72.7%) were assigned ST1 and thus ST1 was the dominant ST for these samples in this phylogroup with the other three samples assigned a different ST each. There was not a ST in the *T. phagedenis* DD treponeme phylogroup which showed obvious dominancy over the other STs, as seen for the *T. medium* phylogroup, although ST 21 was identified in 6/38 (15.8%) samples. Additionally, 7/16 STs identified for the *T. phagedenis* DD treponeme phylogroup were represented by one sample only.

Interestingly, where DNA extract from both tissue and culture were used for the same sample the STs obtained were not the same; for example, culture from healthy foot sample 495 (495C) produced *T. phagedenis* DD treponeme phylogroup ST2 whereas DNA extracted direct from the tissue (495T) produced ST17 (Table 5.5). Furthermore, with regards to culture, aliquots of the same cultures were analysed by MLST which were taken at different times during the period of culturing and in these cases the same STs were not obtained as demonstrated by 568C and 568.1C which produced *T. phagedenis* phylogroup STs 39 and 21 respectively (Table 5.5).

Table 5.5: T. phagedenis DD treponeme phylogroup sample ST summary

Sample ID[a]	Tissue type	MLST allele							ST
		adk	*gdh*	*glpK*	*groEL*	*pyrG*	*recA*	*rplB*	
523C	Foot	1	1	1	1	1	1	1	**1**
N1	Gingiva	1	1	1	1	1	1	1	**1**
N3	Dorsal sac rumen	1	1	1	1	1	1	1	**1**
N4	DD lesion	1	1	1	1	1	1	1	**1**
379T	Gingiva	1	1	1	1	1	9	1	**2**
495C	Foot	1	1	1	1	1	9	1	**2**
587C	Foot	1	4	1	3	1	9	1	**3**
495T	Foot	1	2	1	3	1	9	1	**17**
571T	DD lesion	1	2	1	3	1	9	1	**17**
494T	Foot	2	2	1	3	1	9	1	**20**
204C	Gingiva	1	1	1	3	1	9	1	**21**
381T	DD lesion	1	1	1	3	1	9	1	**21**
384T	RAJ	1	1	1	3	1	9	1	**21**
537C	Gingiva	1	1	1	3	1	9	1	**21**
568.1C	Foot	1	1	1	3	1	9	1	**21**
523.1C	Foot	1	1	1	3	1	9	1	**21**
320C	Foot	2	1	1	1	1	9	1	**22**
492T	Gingiva	2	1	1	1	1	9	1	**22**
529C	Gingiva	2	1	1	1	1	9	1	**22**
573T	Gingiva	2	1	1	1	1	9	1	**22**
645C	Gingiva	2	1	1	1	1	9	1	**22**
517C	Gingiva	1	1	1	3	1	1	1	**27**
682C	RAJ	1	1	1	3	1	1	1	**27**
N2	Reticular pillar	1	1	1	3	1	1	1	**27**
252T	Foot	1	3	1	3	1	1	1	**36**
253C	Foot	1	3	1	3	1	1	1	**36**
663C	Foot	1	3	1	3	1	1	1	**36**
490T	Foot	2	1	1	3	1	9	1	**37**
325.1C	Foot	2	5	1	1	1	1	1	**38**
567T	Foot	1	3	1	3	1	9	1	**39**
568C	Foot	1	3	1	3	1	9	1	**39**
60C	Gingiva	1	3	1	3	1	9	1	**39**
321.1C	Foot	1	3	1	1	1	9	1	**40**
1C	Faeces	1	6	1	3	1	9	1	**41**
321C	Foot	2	3	1	1	1	9	1	**42**
325C	Foot	1	5	1	1	1	9	1	**43**
657T	Gingiva	1	5	1	1	1	9	1	**43**
688C	Foot	1	5	1	1	1	9	1	**43**

[a] *T denotes DNA extract from tissue sample and C denotes DNA extract from culture sample*

Table 5.6: T. pedis DD treponeme phylogroup sample ST summary

Sample ID[a]	Tissue type	MLST allele							ST
		adk	*gdh*	*glpK*	*groEL*	*pyrG*	*recA*	*rplB*	
573T	Ginigva	1	4	3	4	1	5	4	**8**
688C	Foot	4	1	3	3	4	5	1	**9**
494T	Foot	1	4	3	4	6	1	3	**10**
452T	Ginigva	4	4	4	3	5	1	3	**11**
495T	Foot	4	4	1	3	4	6	1	**13**
663C	Foot	4	4	1	3	4	6	1	**13**
490T	Foot	4	4	1	3	1	6	3	**14**
204C	Gingiva	1	4	1	3	1	6	3	**15**
517C	Gingiva	4	4	1	1	6	6	3	**16**
721T	Gingiva	4	1	3	1	1	6	3	**17**
60C	Gingiva	4	1	1	1	1	1	1	**18**

[a] *T denotes DNA extract from tissue sample and C denotes DNA extract from culture sample*

5.3.3.3 Loci Evolution

Full recombination events were not observed between any of the three DD treponeme phylogroups (*T. medium, T. phagedenis* and *T. pedis*). However, within each DD treponeme phylogroup, recombination appeared to play an important role in ST divergence as indicated by split decomposition analysis. The highest levels of recombination within the individual phylogroups occurred for the *T. phagedenis* and *T. pedis* DD treponeme phylogroups with recombination occurring in a much lower extent in the *T. medium* phylogroup (Appendix B).

There was no evidence of positive selection pressures on any of the loci for each DD treponeme phylogroup. However, negative selection pressure was observed for loci (including *adk, pyrG* and *rplB*) in the *T. medium and T. pedis* DD treponeme phylogroups as described previously for Clegg et al., (2016b) (data not shown).

5.3.3.4 Molecular epidemiology of DD treponemes in different tissue types

Tissue types for which STs were obtained include gingiva, healthy feet (plantar aspect between the bulbs of the heel, adjacent to the interdigital cleft where a lesion would normally be found), RAJ, rumen reticular pillar, rumen dorsal sac and DD lesions. These samples types were compared to STs obtained for DD treponemes isolated from DD lesions from multiple species in an earlier DD treponeme MLST study (Clegg et al., 2016b) (Figures 5.11-5.13)

T. medium DD treponeme phylogroup STs obtained for gingiva samples were all ST1 and were therefore situated within the maximum likelihood tree alongside the DD lesion isolates that were also ST1 (Figure 5.11) (Clegg et al., 2016b). For the 11 *T. phagedenis* gingiva samples (tissue and tissue culture) there was more variation with seven different STs (ST1, 2, 21, 22, 27, 39 and 43) identified. Of those identified, STs 1-27 are also found in DD lesions either in sheep, elk or dairy cattle depending upon the ST (Figure 5.12). ST39 and 43 on the other hand have not previously been identified. However, they have also been found in healthy foot tissues in this study. The most predominant *T. phagedenis* phylogroup ST in gingiva was ST22 with 4/11 gingiva containing this ST. ST21 was also present in two gingiva samples, whereas other STs were present in a single gingiva sample. With regards to the *T. pedis* phylogroup, all six gingiva samples (tissue and culture) bore a different ST (ST8, 11, 15, 16, 17 and 18) (Figure 5.13). Each of the gingiva *T. pedis* STs are new and have not been identified before in any tissue type.

Only two RAJ samples, each from a different cow, were analysed by MLST and these only contained treponemes from the *T. phagedenis* DD treponeme phylogroup. Two different STs were obtained, ST21 and ST27 (Table 5.5). ST21 as partially described above has also in this study been found in a lesion, two gingiva and one healthy foot as well previous DD lesions (Figure 5.12). In this study, in addition to the RAJ, ST27 was also found in the gingiva and interestingly a rumen reticular pillar sample. Conversely ST27 has also previously been identified in DD lesions from a dairy cow and sheep (Figure 5.12).

Of the healthy foot tissues that underwent MLST specific for the *T. medium* DD treponeme phylogroup, there were four STs identified (ST1, 4, 12 and 13) (Table 5.4). Whilst ST1 and ST4 have been previously identified in DD lesions (ST1: dairy cows and sheep; ST4: dairy cows) (Figure 5.11); ST12 and 13 are new STs identified in this study and have not been identified in any other tissue type and were only present in a single foot each. There were 14 STs (ST1, 2, 3, 17, 20, 21, 22, 36, 37, 38 ,39, 40, 42 and 43) identified with the *T. phagedenis* phylogroup specific MLST in foot tissues that had been classified as healthy (Table 5.5). Of the 14 STs, seven were new (ST36-43) and not previously identified in a DD lesion (Figure 5.12). ST37, 38, 40 and 42 were present in a single foot and not in any other tissue type. With regards to healthy foot samples that underwent the *T. pedis* specific MLST, four STs (ST9, 10, 13 and 14) were identified with only one ST (ST13) present in more than one foot (2/5 feet) (Table 5.6). All the *T. pedis* STs were new, as previously described in Section 5.3.3.2 and each of the four STs have not been seen in any other tissue type except healthy feet.

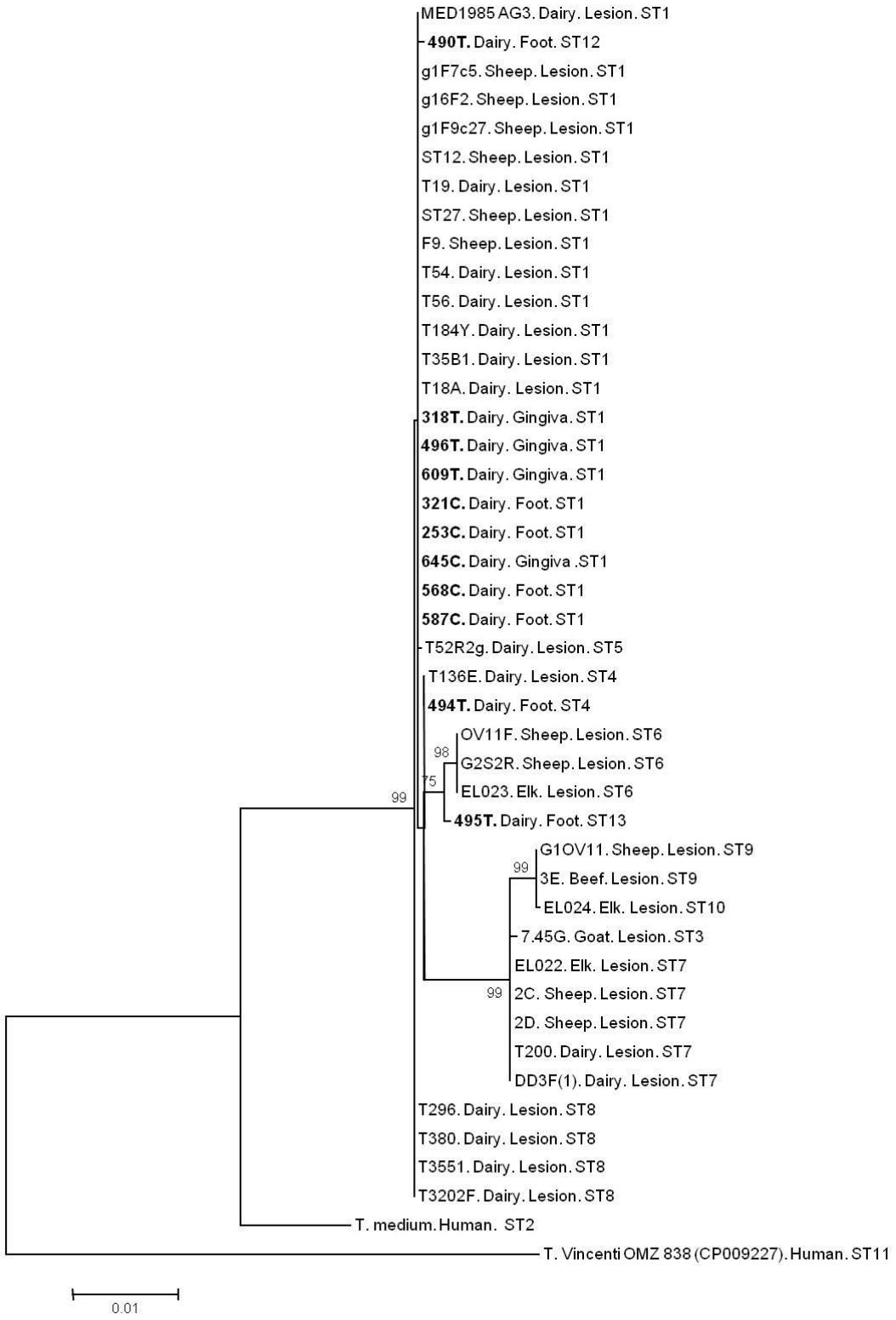


Figure 5.11: T. medium DD treponeme phylogroup concatenated DNA phylogenetic tree based on seven housekeeping genes

*Samples from this study are in **bold**. Samples not in bold are from the Clegg et al., (2016b) study. Each sample is labelled with the sample ID, the host the sample belongs to (dairy/beef cow, goat, sheep, elk or human, the tissue type of the sample (note lesion refers to a DD lesion and foot refers to a healthy foot) and the ST (Bootstrap values below 70 were removed for clarity).*

5.3.3.5 ST presence in more than one anatomical site of the same cow

Where possible, tissue and cultures originating from different anatomical sites of the same cow that were DD treponeme positive by PCR were submitted for MLST to determine whether the same DD treponemes were present in more than one tissue type/ anatomical site of an individual cow. Table 5.7 summarises the results of multiple tissues analysed from individual cows for a total of eight cows. It must be noted that although DD treponemes were present in multiple anatomical sites for some cows, the DD treponemes identified may not have been from the same DD treponeme phylogroup between anatomical sites thus limiting comparison. Furthermore, some anatomical sites may have had multiple DD treponeme phylogroups present.

Of the eight cows in which multiple samples from the same cow were analysed, four cows had an ST that was in more than one anatomical site of the individual cow (Table 5.7). For two of the cows (Cow 1 and 6) in which healthy foot tissue from each of the hind feet was analysed, the same *T. phagedenis* phylogroup ST was found in each foot with cow 1 having ST36 in each foot and cow 6 having ST39. Conversely, cow 5 in which both hind feet were also analysed by MLST for all three DD treponeme phylogroups, did not produce the same STs between the two feet for any of the phylogroups.

A couple of cows had the same ST present in the GI tract that was also present in the DD lesion of that animal. Cow 3 (Table 5.7) had a DD lesion and RAJ sample analysed with the *T. phagedenis* phylogroup specific MLST and ST 21 was identified in both tissue types. Additionally, *T. phagedenis* phylogroup ST1 was identified in the gingiva, rumen dorsal sac and DD lesion of cow 8 (Table 5.7); although it was ST27 that was found in the rumen reticular pillar of that animal. However, another cow in which a gingiva and lesion was analysed by MLST for the same phylogroup had different STs in the two tissue types. Different STs were also observed for two other cows where gingiva and one or more healthy feet samples were analysed per animal for the same phylogroup.

Cows 2, 4, 5 and 7 had different STs observed in each anatomical site for each individual animal (Table 5.7). For example, in cow 5 *T. medium* ST4 was detected in one foot, ST13 in the other foot and ST1 in the gingiva. Furthermore, there were no shared STs between the hind feet of cow 5 for the *T. phagedenis* or *T. pedis* DD treponeme phylogroups either.

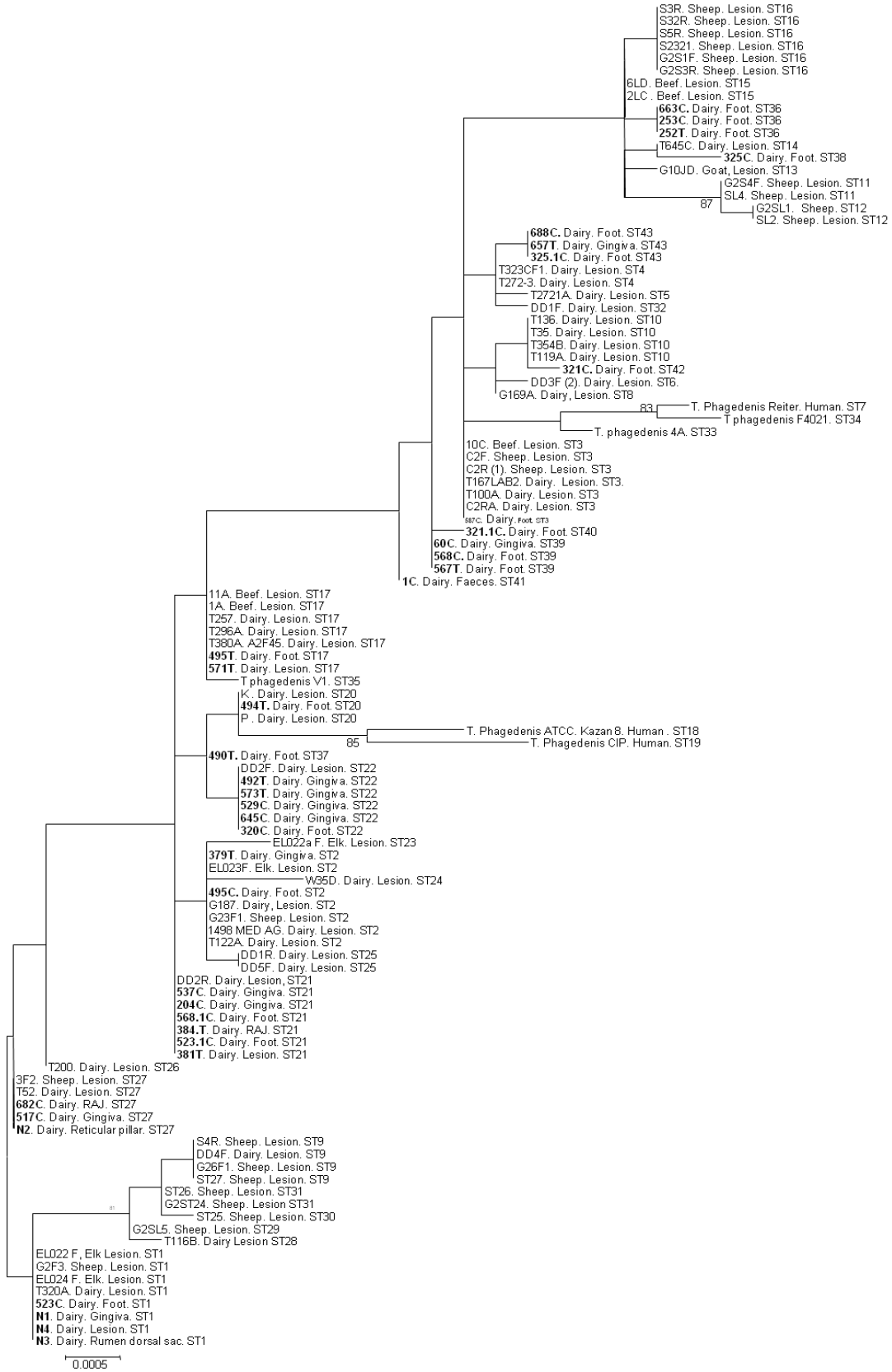


Figure 5.12: T. phagedenis DD treponeme phylogroup concatenated DNA phylogenetic tree based on seven housekeeping genes

*Samples from this study are in **bold**. Samples not in bold are from the Clegg et al., (2016b) study. Each sample is labelled with the sample ID, the host the sample belongs to (dairy/beef cow, goat, sheep, elk or human, the tissue type of the sample (note lesion refers to a DD lesion and foot refers to a healthy foot) and the ST (Bootstrap values below 70 were removed for clarity).*

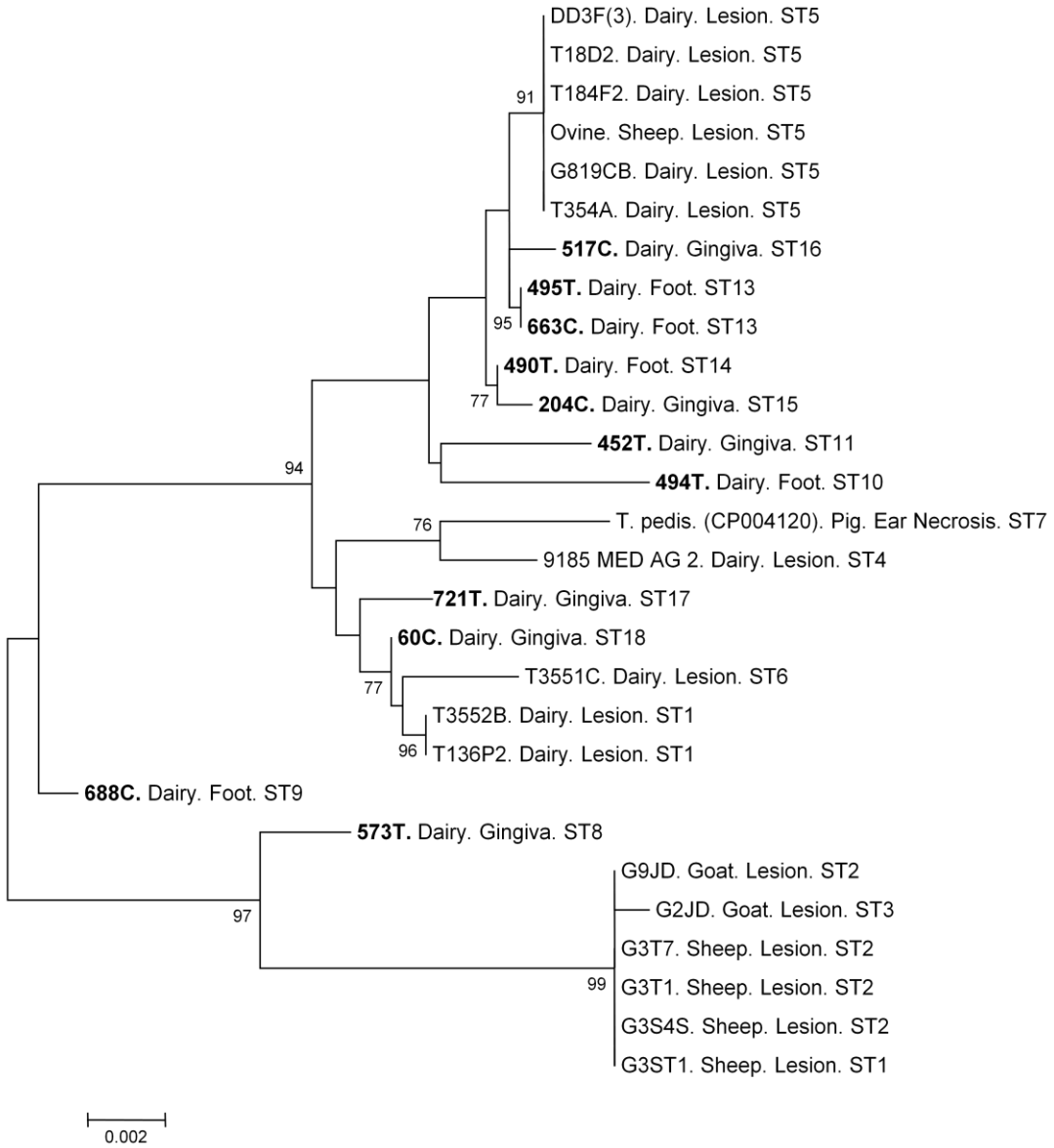


Figure 5.13: T. pedis DD treponeme phylogroup concatenated DNA phylogenetic tree based on seven housekeeping genes

*Samples from this study are in **bold**. Samples not in bold are from the Clegg et al., (2016b) study. Each sample is labelled with the sample ID, the host the sample belongs to (dairy/beef cow, goat, sheep, elk or human, the tissue type of the sample (note lesion refers to a DD lesion and foot refers to a healthy foot) and the ST (Bootstrap values below 70 were removed for clarity).*

Table 5.7: Summary of STs found within different tissues of individual cows

Cow no.	Sample ID[a]	Tissue[b]	Phylogroup[c]	Allelic profile[d]	ST
1	252T	HL Foot	2	1,3,1,3,1,1,1	36
	253C	HR Foot	1	1,1,1,1,1,1,1	1
	253C	HR Foot	2	1,3,1,3,1,1,1	36
2	320C	HL Foot	2	2,1,1,1,1,9,1	22
	321C	HR Foot	1	1,1,1,1,1,1,1	1
	321C	HR Foot	2	2,3,1,1,1,9,1	42
	321.1C	HR Foot	2	1,3,1,1,1,9,1	40
3	381T	HL Lesion	2	1,1,1,3,1,9,1	21
	384T	RAJ	2	1,1,1,3,1,9,1	21
4	490T	HL Foot	1	1,1,1,1,6,1,1	12
	490T	HL Foot	2	2,1,1,3,1,9,1	37
	490T	HL Foot	3	4,4,1,3,1,6,3	14
	492T	Gingiva	2	2,1,1,1,1,9,1	22
5	494T	HL Foot	1	3,1,1,1,1,1,1	4
	494T	HL Foot	2	2,2,1,3,1,9,1	20
	494T	HL Foot	3	1,4,3,4,6,1,3	10
	495T	HR Foot	1	3,1,1,1,5,1,1	13
	495T	HR Foot	2	1,2,1,3,1,9,1	17
	495C	HR Foot	2	1,1,1,1,1,9,1	2
	495T	HR Foot	3	4,4,1,3,4,6,1	13
	496T	Gingiva	1	1,1,1,1,1,1,1	1
6	567T	HL Foot	2	1,3,1,3,1,9,1	39
	568C	HR Foot	1	1,1,1,1,1,1,1	1
	568C	HR Foot	2	1,3,1,3,1,9,1	39
	568.1C	HR Foot	2	1,1,1,3,1,9,1	21
7	571T	HL Lesion	2	1,2,1,3,1,9,1	17
	573T	Ginigva	2	2,1,1,1,1,9,1	22
	573T	Ginigva	3	1,4,3,4,1,5,4	8
8	N1	Gingiva	2	1,1,1,1,1,1,1	1
	N2	Recticular pillar	2	1,1,1,3,1,1,1	27
	N3	Rumen dorsal sac	2	1,1,1,1,1,1,1	1
	N4	LH Lesion	2	1,1,1,1,1,1,1	1

[a] T denotes DNA extract from tissue sample and C denotes DNA extract from culture sample.

[b] Lesion refers to a DD lesion, foot refers to a healthy foot, HL refers to hind left foot and HR refers to hind right foot.

[c] 1 denotes the T. medium DD treponeme phylogroup, 2 denotes the T. phagedenis DD treponeme phylogroup and 3 denotes the T. pedis DD treponeme phylogroup.

[d] Allelic profile ordered alphabetically in terms of the seven housekeeping genes: adk, gdh, glpK, groEL, pyrG, recA and rplB.

5.3.3.6 MLST of a faecal sample

A bovine faecal culture (1C) was PCR positive for the *T. phagedenis* DD treponeme phylogroup and underwent MLST for this phylogroup. The ST was a new ST for the *T. phagedenis* DD treponeme phylogroup ST41 and was not shared with any of the tissues examined in this study (Table 5. 5).

5.4 Discussion

The presence of DD treponemes in tissues other than DD lesions warranted further investigation into the nature of their association with these tissue types. In this study, histopathology and IHC was used to try and determine whether DD treponemes could be carried without causing changes to the tissue associated with disease in the GI tract and non DD lesion pedal tissue and where in these tissues the DD treponemes localised. Additionally, a proportion of samples positive by PCR for DD treponemes underwent MLST specific for the DD treponeme phylogroups they were positive for to determine whether the same ST types found previously in DD lesions (Clegg et al., 2016b) were responsible for colonisation of these other tissue types or whether there was tissue specific tropism occurring with different strains. Together this knowledge would help to delineate the role of these tissues as DD infection reservoirs.

Polyclonal anti-treponemal rabbit antibodies raised against a cocktail of the three cultivable DD treponemes have successfully been used to visualise DD treponemes tracking down the hair follicles of DD lesions (Evans et al., 2009c) and visualise their presence and localisation in CODD lesions (Angell et al., 2015b), DD in goats (Crosby-Durrani et al., 2016) and bovine pressure sores (Clegg et al., 2016d). However, it was unknown whether these polyclonal antibodies would cross react with commensal GI treponemes. Commensal treponemes have been isolated from the rumen, RAJ and faeces of ruminants (Stanton and Canale-Parola, 1980; Paster and Canale-Parola, 1985; Evans et al., 2012b; Sullivan et al., 2015a). However, they have been shown to be quite different from DD treponemes with different growth requirements, enzymatic activities and lack at least two of the virulence factors present in DD treponemes (Evans et al., 2011b; Newbrook et al., 2017; Staton et al., 2017). Furthermore upon phylogenetic analysis, GI commensal treponemes form a distinct phylogenetic cluster away from the treponemes associated with DD (Evans et al., 2011b). Western blot analysis using the polyclonal anti-treponemal rabbit antibodies in this study demonstrated only a small amount of cross-reactivity with antigens from the newly named

GI commensal treponeme species *T. ruminis* and *T. rectale* when compared to *T. medium* DD phylogroup strain T19 (Newbrook et al., 2017; Staton et al., 2017). Thus commensal GI treponemes appear to share few antigens with DD treponemes. Indeed, the presence of GI treponemes does not appear to affect IHC labelling as samples that were negative for DD treponemes but positive for the *Treponema* genus by PCR were either completely negative for labelling with the polyclonal anti-treponemal antibodies or showed very faint background labelling. Background labelling did not appear to be linked solely to *Treponema* genus positive samples either. Further characterisation of the small number of cross-reacting antigens would be required to determine if they are outer membrane antigens and their importance for colonisation/ survival.

Interestingly, HE staining showed that GI tissues that were PCR positive for DD treponemes showed no abnormalities in the tissue that would indicate infection or tissue damage such as ulceration or severe inflammatory cell infiltrates. This suggests that unlike *T. denticola,* DD treponemes can colonise the GI tract without associated tissue abnormalities. However, DD treponemes were also not visualised in these tissues with the polyclonal anti-treponemal rabbit antibodies and although background labelling occurred in some samples, this type of labelling was also present in the controls that were negative by PCR and culture for DD treponemes. There may be several explanations for why DD treponemes were not labelled. 1) DD treponeme colonisation was not diffuse in area and thus may be have been missed in the sections. 2) DD treponemes may not actually penetrate the tissue but be in the salvia / mucus coating the tissue and were thus lost during tissue processing or 3) there may have only been a very low concentration of DD treponemes present and were thus not picked up by labelling. Only a small number of GI tissues were assessed using IHC (*n*=6) and thus caution must be taken upon interpretation of these results. A larger sample population would be required to provide further understanding of DD treponeme colonisation of GI tissues.

The presence of DD treponemes in 18% of healthy foot tissue (*n*=217) (Chapter 4) was surprising as although a small number of a studies have detected DD treponemes in healthy non-DD lesional foot tissue using molecular techniques (Evans et al., 2009c, 2012b; Rasmussen et al., 2012), the wider consensus is that DD treponemes are predominantly detected in DD lesions with other studies failing to detect DD treponemes in healthy tissue using PCR and finding a very low abundances by deep sequencing (Sullivan et al., 2014b, 2015b, Zinicola et al., 2015a; b; Crosby-Durrani et al., 2016; Nielsen et al., 2016). These

results prompted investigation into whether macroscopically healthy tissue positive by PCR for DD treponemes were indeed healthy or showed abnormalities consistent with infection. Staggeringly, 77.8% of the healthy foot tissues positive by PCR for DD treponemes submitted for histopathology (*n*=36) had abnormalities present in the tissue. The most notable changes in the tissue were irregular thickening of the epidermis as a result of either parakeratotic hyperkeratosis, orthokeratotic hyperkeratosis or both, as well as infiltrates of immune cells (degenerative neutrophils, lymphocytes and macrophages etc) and erosion of the epidermis. Complete removal of the epidermis as a result of ulcerative changes occurred in 13.9% of samples.

Many of these abnormalities described for DD treponeme positive healthy tissues are indicative of disease pathology and are in accordance with DD lesion pathology, thus suggesting that not only can these tissues not be classed as 'healthy' but it is likely that these tissues may be in the very early stages of DD lesion development before normal clinical lesions can be observed by eye. Indeed, another study analysing healthy foot tissue by histopathology found that for the three samples in which DD treponemes were detected there were abnormalities in the tissues consistent with severe hyperplasia of the epidermis, hyperkeratosis and degenerated ballooning keratinocytes (Rasmussen et al., 2012). These changes in combination with the presence of DD treponemes lead to the author redefining these samples as subclinical DD (Rasmussen et al., 2012).

Whilst the majority of samples did show abnormalities in the tissue, there were eight samples in which the tissue appeared healthy with no abnormalities. These tissues were all positive by PCR for at least one or more of the three DD treponeme phylogroups. This does suggest that colonisation may not always be associated with damaged tissue as previously thought. However, it may also be that these tissues were sampled early in infection before damage could occur providing that DD treponemes are involved in disease initiation. What is more, there was no bacterial infiltration observed in any of these tissue samples. However, two of the healthy feet samples (three if the IHC sample only is included) with no abnormalities did label positively for treponemes but with no treponeme morphology. On further inspection the intense labelling for treponemes was either thinly spread or in a small area and was only present on the very surface of the stratum corneum suggesting the treponemes may not have penetrated into the skin yet and perhaps that colonisation was recent.

One of the questions raised by the presence of DD treponemes in healthy tissue was whether this could be the detection of DD treponemes still present in the tissue following the DD lesion resolving. DD treponeme labelling was predominantly at the surface of the stratum corneum and extracellular between the layers of the stratum corneum. In a few examples, the treponemes are tracking down the fissures in the irregular epidermis or the epidermal laminae. This therefore suggests these tissues are newly colonised as if colonisation was from a previous infection DD treponemes would be expected to be deeper in the tissue nearer the stratum spinosum and dermis where DD treponemes have been seen to localise in clinical DD lesions (Dopfer et al., 1997; Moter et al., 1998; Cruz et al., 2005; Klitgaard et al., 2008; Evans et al., 2009c), and not primarily on the very surface of the epidermis. Again, along with evidence of tissue abnormalities the presence of DD treponemes nearer the surface suggests that this may be the very beginning of infection and lesion development and thus DD may be 'subclinical' in these tissues.

It has previously been suggested in terms of CODD and DD lesions in goats that DD treponeme localisation in the superficial surface of the epidermis may facilitate transmission of the disease through sloughing of keratinocytes and other necrotic material (Crosby-Durrani et al., 2016). There were several pedal tissues in this study in which sloughed keratinocytes and serocellular crusts were present and they were largely labelled intensely for DD treponemes. Whilst this does not directly indicate this as a mode of transmission, it does show an association of DD treponemes with this type of material, which could provide a vehicle for transmission in the early stages of infection when treponemes are not as primarily deeply localised in the tissue.

Whilst healthy foot samples negative for DD treponemes by PCR (*n*=4) used as controls all appeared normal with no treponeme labelling present following IHC, one of the 'other' foot tissue samples that was negative for DD treponemes by PCR and showed hyperkeratosis incurred some labelling for treponemes by IHC tracking down into the fissures of the epidermis. It may be that the section of sample taken for PCR and culture missed the treponemal presence demonstrated here. Another 'other' foot tissue which was positive by PCR for at least one of the DD treponeme phylogroups also showed labelling of DD treponemes on the surface layers of the stratum corneum. This sample was characterised by orthokeratotic hyperkeratosis and the presence of multiple intracorneal pustules (absent of labelling). Intracorneal pustules have been associated with CODD lesions where they are present in early stage legions (Angell et al., 2015b). The presence of DD

treponemes whether by PCR or IHC along with abnormalities suggests these tissues could be DD lesions that had an atypical appearance which owed to their classification as 'other'. However, it may also be that DD treponemes have opportunistically invaded these damaged tissues as has recently been described for hock lesions and bovine pressure sores (Clegg et al., 2016a; d). DD treponemes appear to be completely absent from the remaining 'other' foot tissue samples and as DD treponemes are always associated with DD lesions it highly likely that the poor health of these tissues are unrelated to DD.

The molecular epidemiology of DD treponemes in different tissue types was explored using a MLST approach piloted to determine whether tissue tropism was apparent actually within (rather than across) DD treponeme phylogroups. MLST was carried out for the first time on DNA extracts from tissue and/ or culture originating from the gingiva, rumen dorsal sac, reticular pillar, RAJ and healthy hind feet in the region where a DD lesion would normally develop as well as DD lesions. The tissue samples used for MLST were primarily chosen based on whether there were other tissues positive for DD treponemes within that cow. Cultures which appeared to have spirochaete-like morphology, confirmed to be DD treponemes by PCR, were also included. Overall STs were successfully obtained for all 28 tissues and 32 cultures. The number of samples used especially with regards to the GI tissues, were limited to the number of samples positive by PCR for DD treponemes in this study. Archived tissues from one cow in which multiple GI tract tissues were positive were examined to increase the number of tissue types and cows with multiple anatomical sites positive for DD treponemes analysed by MLST. Isolates from DD lesions previously characterised by MLST in terms of host tropism and geographical population structure were included in analysis for this study for comparison with STs obtained from non-DD lesional tissues (Clegg et al., 2016b).

It may be hypothesised that particular DD treponeme STs show tropism for certain tissues based on lack of disease in some tissue types and the varying environmental conditions of these tissues, as has been observed for *Chlamydia pecorum* strains in sheep (Jelocnik et al., 2014). However, many of the STs obtained for the *T. medium* and *T. phagedenis* DD treponeme phylogroups were present in multiple tissue types/ anatomical sites of the dairy cow, as demonstrated by *T. phagedenis* DD treponeme phylogroup ST21 which was found in dairy cow DD lesions both in this study and previously (Clegg et al., 2016b), gingiva, RAJ and healthy foot tissue. Other STs previously reported in multiple hosts were also found in multiple dairy cow tissues suggesting that these STs have both a broad tropism for different

hosts and a broad tropism for different tissues. For example, *T. phagedenis* phylogroup ST1 which has been identified in elk, dairy cow and sheep DD lesions was also found in gingiva, healthy foot and rumen dorsal sac tissue. A lack of tissue tropism has been demonstrated by MLST for other bacterial species including *Chlamydia trachomatis* in humans where the same STs were found in multiple tissue types in women (Versteeg et al., 2014). Thus, it may be possible that potential transmission events (PTEs) are occurring between different tissue types between cows for example a ST from the RAJ may infect the foot of another cow or possibly the same cow.

Some STs appear to have a predilection for appearing in some tissue types compared to other STs (although they do not appear solely in that tissue type). Out of the 13 STs identified to date for the *T. medium* DD treponeme phylogroup, all four gingiva samples examined were ST1. Similarly for the *T. phagedenis* DD phylogroup, four of the eleven gingiva analysed shared the same ST and another two gingiva samples shared another ST, despite the fact there are greater than 40 STs in the *T. phagedenis* phylogroup that could be present. Although these results must be viewed with caution due to the very small sample number of samples analysed, with a larger number of samples, a larger spread of STs may be uncovered in these tissue types.

Hind foot tissues classified as healthy were included in the MLST analysis to elucidate whether STs carried on healthy feet were the same pathogenic STs associated with DD lesions. However, histopathology carried out on healthy foot tissues described earlier in the chapter (Section 5.3.2) demonstrated that very few of these foot tissues were actually healthy and that some could possibly be subclinical DD. In contrast, a small number of healthy foot tissues showed no abnormalities in the tissue indicative of disease and two of these tissues (samples 253 and 688) were submitted for MLST. For sample 253, the *T. medium* and *T. phagedenis* phylogroup STs obtained had previously been identified in DD lesions. Thus, it would appear that STs involved in lesions can colonise healthy feet without disease, although it cannot be said that this foot would not then go on to develop DD presuming this foot was sampled early after colonisation. The two STs (*T. phagedenis* and *T. pedis* phylogroups) obtained from healthy foot sample 688 were novel, with the *T. pedis* phylogroup ST identified in no other samples and the *T. phagedenis* ST also identified in a foot and a gingiva sample from other cows in this study. The presence of novel STs in this healthy foot which have not previously been identified in DD lesions may represent STs which are not disease associated. However, the *T. phagedenis* ST which was identified in

another foot, and although originally classified as healthy, did show signs of tissue abnormalities via histopathological investigation. Furthermore, sample numbers containing these novel STs are very low and further samples will need to be analysed for a definitive answer.

Multiple anatomical sites were analysed by MLST for eight cows with at least one DD treponeme phylogroup represented in more than one tissue. Half of the cows had an ST identified in more than one anatomical site within an individual cow, although not necessarily in all anatomical sites explored for that cow. Thus, ST dissemination between different anatomical sites may be occurring for some cows. Indeed, it could be postulated for one cow in which the DD lesion on the hind foot and the RAJ have the same ST, that the ST in the RAJ may have been passed to the foot through shedding in the faeces. Furthermore, the presence of the same ST in the gingiva and rumen dorsal sac of a cow provides support for the idea that rumen fluid may act as vehicle for DD treponeme dissemination between these sites as discussed in Chapter 4 (Nascimento et al., 2015). However, in each case this is only one cow and a greater population size of cows would need to be studied to substantiate these theories. The other four cows, however, had STs unique to each anatomical site suggesting within cow dissemination of STs does not always occur or that multiple STs may co-populate a cow at one time leading to differences in detection between sites.

Interestingly, where a tissue was analysed by MLST more than once, for example using 1) the tissue and the culture of a section of the same tissue sample for typing or 2) multiple culture aliquots from different time points in the culture incubation, the same STs were not obtained. This is unsurprising as DD lesions are polytreponemal with a high percentage of lesions containing more than one of the three cultivable DD treponeme phylogroups within the DD lesions as determined using a PCR approach, and metagenomic studies have uncovered a plethora of treponemes in lesions (Evans et al., 2009c; Krull et al., 2014; Zinicola et al., 2015a; b; Nielsen et al., 2016). Furthermore, anecdotally, isolation of pure DD treponeme isolates can be very difficult due to different phylogroup and strain growth being difficult to separate. It is therefore highly like that more than one ST from the same or different DD treponeme phylogroup is within a tissue and that in certain conditions different STs may dominate others resulting in their detection. Additionally some STs detected, using tissue MLST but not in culture MLST of the same sample, may not be very cultivable and may either be easily outcompeted or conditions are not right for their

growth resulting in their absence in culture. Thus it may be that within a cow the same STs are present in different anatomical sites but either detection methods used for each tissue has lead to different STs detected or another ST may be more abundant in that site at the time of sampling compared to other sites and it could be postulated that this is as a result of being outcompeted by an ST better suited to that tissue type niche.

The addition of non-DD lesional samples to the MLST data of DD lesions previously analysed had little effect on the amount of variation seen within loci compared to the previous study (Clegg et al., 2016b). However, there was a greater range in variation of the *T. pedis* DD treponeme phylogroup housekeeping gene sequences as a result of greater sequence variability at the *pyrG* locus, rising from 4% variation to 7% variation in sequences. Indeed, the *T. pedis* DD treponeme STs obtained were all novel STs and in two cases this was as a result of a novel allele at the *pyrG* loci. Two novel alleles were also observed at the *pyrG* locus in the *T. medium* DD treponeme phylogroup housekeeping genes. Thus, the mutation rate may be higher in this locus than any of the others. In addition, as previously described by Clegg et al., (2016b), there were a high number of recombination events observed in the *T. phagedenis* and *T. pedis* DD treponeme phylogroups that appears to drive the production of new STs.

There was a large number of novel STs unearthed in this study from non-DD lesional tissue types, although the majority of the novel STs were singletons. This was particularly evident in the *T. pedis* DD treponeme phylogroup which has previously been suggested to be evolving more rapidly (Clegg et al., 2016b). Furthermore, these novel STs may indicate that there are STs only present in non-DD lesional tissue. However, the sample numbers used in this study were limited and thus further investigation into the molecular epidemiology of DD treponemes within different tissue types is warranted.

In this study, MLST was carried out on tissues and cultures of tissues as opposed to isolated DD treponemes from cultures, as originally carried out for DD lesions (Clegg et al., 2016b), due to the difficulties in obtaining isolates from these tissue types. MLST directly from samples seemed to be successful with STs obtained for all DNA extracts from tissues and cultures that underwent MLST. The presence of multiple strains of DD treponemes in tissues where DD treponemes have been detected and the high number of novel STs found here does raise the question as to whether the seven alleles in an allelic profile are all from the same bacteria or whether different genes were sequenced from different DD treponemes present in the sample, thus these results should be considered with caution.

However, no non-specific amplification was observed and upon analysis of the nucleic acid sequence chromatograms, there was no evidence of double peaks to indicate that different gene copies were picked up when sequenced. Additionally, the identification of previously known STs for *T. medium* and *T. phagedenis* DD treponeme phylogroups suggests that allelic profiles obtained were from only one treponeme. Furthermore, DNA extracts direct from tissue swabs have been successfully used to carry out MLST for *C. pecorum* in sheep (Jelocnik et al., 2014) and more recently MLST carried out directly on human clinical tissue samples enabled the typing of *Leptospira spp.* (Weiss et al., 2016).

A problem encountered for the development of MLST direct from tissues for *Leptospira spp.* was that the assay had low limits of detection compared to MLST carried out on isolates (varied with sample type) which hampered the acquirement of full allelic profiles (Scola et al., 2006; Agampodi et al., 2013; Weiss et al., 2016). This may have been due to the sample type matrix or variance in bacterial abundance between tissue types although, in a later study, nesting of the MLST primers vastly improved the number allelic profiles obtained (Weiss et al., 2016). However, in the current study full allelic profiles were obtained for all samples independent of type. Further investigation into the validity of direct MLST from tissue for DD treponemes is required to dispel any ambiguity. Furthermore, a direct comparison of MLST and DD treponeme phylogroup PCR assays directly on tissue may be warranted to determine which method is more sensitive for treponeme detection.

Interestingly, the application of the DD treponeme specific PCR assays to cultures containing spirochaete-like morphology prior to MLST enabled the detection of DD treponemes in samples that were negative for DD treponemes by direct PCR of the tissue. An example of which is RAJ culture sample 682 which was positive for the *T. phagedenis* DD treponeme phylogroup by PCR but negative when the tissue was directly analysed. Thus, this increases the detection rate of DD treponemes in the RAJ in Chapter 4 from 0.8% to 1.7%. Furthermore, six additional gingiva samples were positive for DD treponemes following PCR analysis of their respective cultures in which spirochaete-like morphology was observed; raising the detection rate of DD treponemes in gingiva from 6.6% to 11.5%. Two more healthy feet were identified as DD treponeme positive by this method resulting in a detection rate of 18.9% (previously 18%) and two 'other' foot tissues were PCR positive for DD treponemes in culture raising the percentage positive from 12.5% to 25%. (See Chapter 4 for full details of previous results).

Remarkably, DD treponemes were detected in bovine faeces via PCR of the faecal culture in which spirochaetes were observed. The faecal sample itself had previously tested negative for DD treponemes via direct PCR on the sample. Prior to this project DD treponemes have only ever been detected in faeces using deep sequencing techniques but in Chapter 4 a treponeme belonging to the *T. phagedenis* DD treponeme phylogroup was isolated from a bovine faecal culture and in this study DNA from the *T. phagedenis* DD treponeme phylogroup was detected by DD treponeme specific PCR assays of a different faecal culture. MLST was carried out on this sample to see if it contained an ST previously seen in DD lesions. However, a novel ST was obtained that was present in the faecal sample only. It may be this ST is specific to faecal samples. Further investigation of larger faecal sample numbers would be required. Based on the increased sensitivity in detection compared to tissues it would be pertinent to include DD treponeme phylogroup specific PCR of cultures as routine for sample investigation; in some studies this method is already implemented (Clegg et al., 2016d; e; Angell et al., 2017).

In conclusion, with regards to the GI tract samples, DD treponemes with STs that are either novel or found in DD lesions appear to be able to colonise GI tissues without typical changes in the tissue associated with disease from a histopathological perspective. Thus, so far there appears to be little evidence of tissue tropism for DD treponemes STs. Tissue from the plantar aspect of the foot between the bulbs of the heel and adjacent to the interdigital cleft which did not appear to be macroscopically DD-affected but were DD treponeme positive by PCR/ culture were found to have histopathological changes present that could be indicative of disease development or subclinical DD. Infection of DD treponemes within these foot tissues appears to be new as opposed to re-emerging from resolved lesions and many have STs also found in DD lesions. Additionally, not all cows which were positive for DD treponemes in multiple anatomical sites/ tissue types had the same ST throughout. This data gives important insight into the microbial ecological dynamics of DD treponeme carriage in non-DD lesional tissue. In particular, it highlights the importance of these sites as DD treponeme infection reservoirs due to their ability to carry DD lesion associated STs. These observations warrant further surveillance of these non-DD lesion tissue types and studies into how farm management can be used to prevent associated disease transmission to reduce DD transmission on farms in the future.

Chapter 6: DD treponeme survival under different host and farm environment conditions

6.1 Introduction

Bacteria belonging to *Treponema* are described as anaerobic (or occasionally microaerophillic), fastidious, spiral and highly motile due to their unique periplasmic flagella arrangements (Brenner et al., 1984). Members of this spirochete genus are typically host associated and found in human, insect and animal tissues. Treponemes have a wide host and tissue range including the oral cavity, gastrointestinal (GI) tract, genital tract and hoof, with the majority of these environments being extremely dynamic in nature (Brenner et al., 1984; Lilburn et al., 1999; Radolf and Lukehart, 2006). Treponemes may be commensal as with *T. bryantii* and the recently named *T. ruminis* which are considered symbionts of the bovine rumen (Stanton and Canale-Parola, 1980; Newbrook et al., 2017) or *T. phagedenis* which is a commensal of the human genital tract (Brenner et al., 1984). Additionally there are pathogenic treponemes, for example, *T. pallidum* subsp. *pallidum* the causative agent of syphilis in humans (Noordhoek et al., 1990). In animals, particularly livestock, treponemes are considered to have a key pathogenic role in DD lesion development and progression (Choi et al., 1997; Stamm et al., 2002; Evans et al., 2008; Klitgaard et al., 2008).

The complex growth requirements of treponemes have made this genus difficult to culture and isolate, and only a small fraction of treponemes reported through molecular techniques have been cultivated. Indeed, *T. pallidum* subsp. *pallidum* the most widely studied treponeme is uncultivable and only survives within a living host (Radolf et al., 2016). However, the pathogenic treponemes associated with DD, which are the focus of this study, have been successfully cultivated from DD and CODD lesions in various animal species (Walker et al., 1995; Stamm et al., 2002; Trott et al., 2003; Evans et al., 2008; Sullivan et al., 2013, 2014b, 2015b; Clegg et al., 2015). In the UK and USA characterisation of treponemes isolated from DD lesions has lead to the identification of three distinct cultivable DD phylogroups known as the *T. medium* phylogroup, *T. phagedenis* phylogroup

and *T. pedis* phylogroup (Stamm et al., 2002; Evans et al., 2008, 2009b). The DD treponemes demonstrated good growth under anaerobic conditions at 36-40 °C in culture media or agar (designed for fastidious microorganisms, pH 7) supplemented with serum/ blood (Evans et al., 2008, 2009c; Wilson-Welder et al., 2013). The three phylogroups differ in their growth time with the *T. medium* phylogroup requiring the longest incubation to reach peak growth (9-10 days) and the *T. pedis* phylogroup requiring the least (3-4 days) (Evans et al., 2008). Furthermore, distinct differences could be observed between each of the three DD treponeme phylogroups with regards to the size/ morphology of the treponemes as well as their colonies, enzymatic profiles and 16S rRNA gene sequences (Stamm et al., 2002; Trott et al., 2003; Evans et al., 2008). Additionally comparison of bovine GI commensal treponemes with the DD-associated pathogenic treponemes identified the DD treponemes as both phenotypically and genotypically distinct (Evans et al., 2011b).

The identification of DD treponemes in different host tissues and the environment by molecular and cultivation approaches calls into question what we know about the survival ability of DD treponemes under the conditions presented in these potential reservoirs (Evans et al., 2012b; Klitgaard et al., 2014, 2017; Sullivan et al., 2014a; Nascimento et al., 2015; Zinicola et al., 2015b). In these reservoirs DD treponemes would be exposed to different nutrient availabilities, different oxygen concentrations, temperatures and pHs; for example on fomites such as foot trimming knife blades there would be limited nutrients, higher concentrations of oxygen and a lower temperature than would be found in the host. Based on current knowledge about DD treponeme growth requirements, it could be suggested that survival would be poor under these differing conditions (Evans et al., 2008, 2009b; Wilson-Welder et al., 2013).

The ability to cultivate DD treponemes enables the differentiation between detection and viability when investigating DD treponeme infection reservoirs. Understanding DD treponeme viability in potential infection reservoirs is necessary to determine the importance of these reservoirs for enabling transmission on and between farms. Thus understanding DD treponeme survival and longevity in certain environments can inform upon improved targeted control strategies for DD.

There is a dearth of studies investigating the survival of DD treponemes under varying conditions in relation to where infections reservoirs may be found. A recent study by Angell et al., (2017) investigated the survival of DD treponemes on sterile disposable gloves

through DD treponeme PCR and culture of swabs taken from gloves, used to handle a CODD-affected foot, for five consecutive days following handling. The *T. medium* DD treponeme phylogroup remained viable on gloves for one day whereas the *T. phagedenis* and *T. pedis* DD treponeme phylogroups remained viable for three days on gloves. However, by PCR all three DD treponeme phylogroups could be detected for the full five day duration (Angell et al., 2017). This study suggests treponemes are able to survive and remain for a short period of time in aerobic conditions containing little nutrients, although their presence may be detected for longer if the study was longer in duration. Treponeme survival under different temperature and pH ranges has previously been investigated for culturing conditions (Brenner et al., 1984; Wilson-Welder et al., 2013). However, to the author's knowledge, only one study published findings for DD treponemes specifically. This study found that optimal growth of a *T. phagedenis* DD treponeme phylogroup isolate was at 40 °C (range of 29-42 °C) in media that was pH 7.4 (range of pH 6.5-8) (Wilson-Welder et al., 2013).

Studies investigating the survival of bacteria, other than treponemes, have employed microcosms to replicate certain environmental conditions. The survival of *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* (the causative agents of swine dysentery and porcine intestinal spirochaetosis) in soil, faeces and soil containing faeces has been investigated by creating microcosms of these conditions and spiking the bacteria into them with survival monitored by regular subculturing into optimal conditions for these *Brachyspira* spp. growth and PCR assays (Boye et al., 2001). A similar method has been used to determine the survival of *Dichelobacter nodosus,* the causative agent of ovine foot rot, in different soil types (Muzafar et al., 2016). Furthermore this method has also been used to determine the survival of *Escherichia coli* in different types of bedding typically used in dairy operations (LeJeune and Kauffman, 2005; Westphal et al., 2011).

To gain further insight into the survival of DD treponemes within infection reservoirs, DD treponemes will be subjected to a range of conditions (via microcosms and other methods) which may affect growth and survival either in the host or in the dairy farm environment in order to delineate the temporal viability of these potential infection reservoirs. Therefore the aims of this study are to determine the growth and survival of DD treponemes in 1) a range of temperatures under anaerobic conditions 2) a range of pHs under anaerobic conditions at 36 °C; 3) faeces under aerobic conditions at 12 °C and 4) different bedding types in aerobic conditions at 12 °C.

6.2 Materials and methods

6.2.1 DD treponeme cultivation

Cultures of *T. medium* DD treponeme phylogroup strains T19 and T56, *T. phagedenis* DD treponeme strains T320A and T354B and *T. pedis* DD treponeme phylogroup strains T3552B and T3554A were grown from stocks and maintained as described in Chapter 2 Section 2.5.1 and subjected to multiple passages prior to study initiation to ensure strains were growing optimally and consistently. Furthermore strains were subjected to DD treponeme phylogroup specific assays (Chapter 2 Section 2.7.2) regularly to ensure the correct phylogroup was present. The strains were previously isolated and sequenced from DD lesions and archived by this laboratory (Evans et al., 2008).

6.2.2 Faeces and bedding sample collection

Faecal samples were collected as described in Chapter 2 Section 2.4 from DD-unaffected low milk yield and high milk yield cows from farm A (Chapter 2 Table 2.3). Following collection faecal samples were stored at -20°C and were later pooled before microcosm set up.

A total of five different types of unused bedding were collected: sand and sand 5% (w/w) lime (calcium oxide) were collected from Farm F, wheat straw was collected from Farm B, recycled manure solids (RMS) was collected from Farm C and sawdust was collected from Farm G (Chapter 2 Table 2.3). Straw was chopped into approximately 5 mm^2 pieces for ease of handling. Samples were stored at 4°C prior to microcosm preparation.

6.2.3 DD treponeme survival in faeces and bedding microcosms

Microcosm experiments were carried out in triplicate on separate days and within each experiment there were three replicates of each microcosm. Microcosms were omitted from further analysis if contamination with other bacteria was observed. Figure 6.1 depicts a summary of the microcosm methodology.

Both bedding and thawed pooled faeces were sterilised using an autoclaving protocol of 126°C for 20 minutes. Following sterilisation the pooled faeces was rehydrated with sterile ddH_2O to the weight the sample was prior to autoclaving, then 1 g of the rehydrated sterile pooled faeces was weighed into 2 ml tubes. Each type of sterile bedding was also weighed

(1 g) into universal tubes (straw, RMS and sawdust) or 2 ml tubes (sand and sand 5% (w/w) lime) depending upon volume.

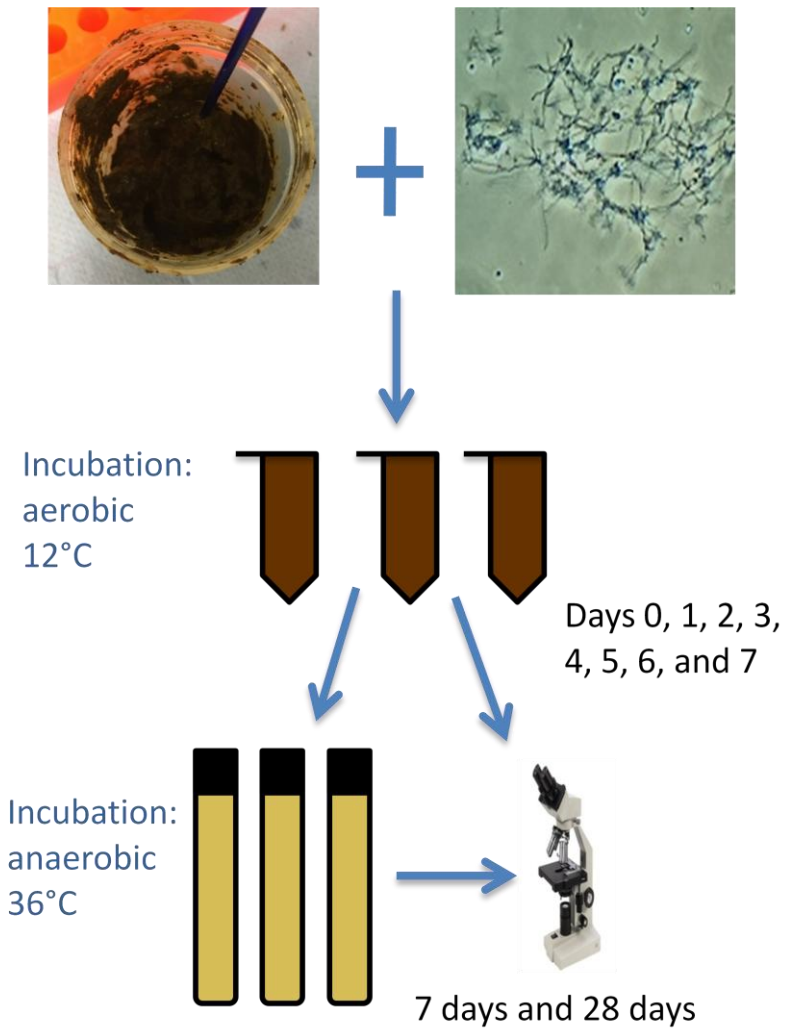


Figure 6.1: Pictogram of DD treponeme survival experiment procedure

Pictogram describing experimental procedure for investigation into the survival of DD treponemes in faecal microcosms (which can also be applied bedding microcosms). Faecal microcosms were spiked with either T. medium phylogroup strain T19, T. phagedenis phylogroup strain T320A or T. pedis phylogroup strain T3552B in triplicate. Faecal microcosms were aerobically incubated at 12 °C for 7 days. On each of the 7 days the microcosms were inoculated into culture medium enriched for treponemes and growth and motility scored by phase contrasts microscopy. Microcosms inoculated into media were then growth and motility scored by phase contrast microscopy after 7 days and 28 days anaerobic incubation at 36 °C.

For each DD treponeme phylogroup strain (*T. medium* phylogroup strain T19, *T. phagedenis* phylogroup strain T320A, *T. pedis* phylogroup strain T3552B) cultures were checked by phase contrast microscopy for growth and purity, then pooled and absorbance was measured at 540nm. Pooled cultures were concentrated and split so that half were resuspended in 1X PBS (pH 7.4; Chapter 2 Table 2.2) for inoculation into the microcosms containing either faeces or bedding and the other half were resuspended in their respective culture media to act as a control. After ten-fold dilution of the stock inoculums, ODs obtained were 0.07 for T19, 0.255 for T320A and 0.25 for T3552B corresponding to final concentrated inoculums of 6.5×10^8, 3.9×10^9 and 3.2×10^9 cells/ml respectively. These ODs were chosen as they enabled easy visualisation of the treponemes in the microcosms and resulted in a large enough concentration of treponemes that growth would be expected when the subsequent volume of bedding or faeces was sub-cultured into the bacterial growth medium.

Faecal microcosms were inoculated in triplicate with 500 µl of one of the three DD treponeme phylogroups resulting in three microcosms of *T. medium* phylogroup strain T19, *T. phagedenis* phylogroup strain T320A and *T. pedis* phylogroup strain T3552B. Whereas each of the five types of bedding were inoculated in triplicate with 500 µl of the *T. phagedenis* phylogroup T320A strain only. Control microcosms were also prepared by inoculating 500 µl of each strain (in media) for the faeces experiments or strain T320A only for the bedding experiments into an empty 1.5 ml tube in triplicate. Each of the strains were also inoculated (~30 µl) into OTEB 10% (v/v) FCS or RS depending upon phylogroup requirements (Chapter 2 Table 2.1) and incubated as described in an anaerobic cabinet (85% N_2, 10% H_2 and 5% CO_2, 36 °C) as a control, which ensured that strains were still able to grow in optimum culture conditions following preparation. Negative microcosms were set up in triplicate which contained either faeces or each bedding type only without inoculation of DD treponemes.

Following inoculation each microcosm was mixed well and allowed to incubate aerobically at 12°C. Samples were collected from microcosms on day 0 (~15 minutes post inoculation) and then on each day for 7 days in total. During collection the microcosm was mixed well and a wet mount of a sample from the microcosm was made for phase contrast microscopy. Treponemes were scored for growth and motility in each microcosm using the system described in Table 6.1. A 30 mg sample of bedding/faeces from each microcosm was taken for inoculation into OTEB 10% (v/v) RS for *T. medium* phylogroup strain T19 and

OTEB 10% (v/v) FCS for *T. phagedenis* phylogroup strain T320A and *T. pedis* phylogroup strain T3552B. Microcosms inoculated into media were allowed to incubate in an anaerobic cabinet (85% N_2, 10% H_2 and 5% CO_2, 36 °C) and scored for growth and motility via phase contrast microscopy after 7 and 28 days incubation.

Table 6.1: Description of growth and motility scores

	Description	
Score	**Growth**	**Motility[a]**
0	No treponemes	No movement, fixed to bottom of slide
1	Only a few treponemes visible (~1-10)	Brownian motion
2	> 10 treponemes visible	< 50% of treponemes motile
3	~50% of slide covered with treponemes	50% of treponemes motile
4	~75% of slide covered with treponemes	75% of treponemes motile
5	Dense growth of treponemes with almost 100% of the slide covered	100% of treponemes motile

[a] *Includes all forms of treponeme motility*

6.2.4 DD treponeme cultivation under different temperatures

Pooled cultures of either *T. medium* DD treponeme phylogroup strain T19, *T. phagedenis* DD treponeme phylogroup strain T320A and *T. pedis* DD treponeme phylogroup strain T3552B were checked by phase contrast microscopy for growth and contamination, and absorbance at 540 nm was measured using a spectrometer. If required, pooled cultures were diluted with culture media to final ODs of 0.25, 0.43 and 0.37 for the *T. medium* phylogroup, *T. phagedenis* phylogroup and *T. pedis* phylogroup corresponding to stock inoculums of 8.75×10^7, 1.14×10^8 and 2.69×10^8 cells/ml respectively. Treponemes were inoculated into 7 ml of OTEB 10% (v/v) serum as described below. The working inoculum concentrations were chosen based on previous work by Evans et al., (2009a).

For each of the three DD treponeme phylogroup strains, using a sterile glass pasteur pipette (VWR International Ltd, Leicestershire, UK), three drops (~90 µl) of the culture was inoculated in triplicate into 7 ml tubes of OTEB 10% (v/v) FCS (*T. phagedenis* and *T. pedis* phylogroups) or OTEB 10% (v/v) RS (*T. medium* phylogroup) (Chapter 2 Table 2.1) for each of the six temperatures investigated. Each of the strains were also inoculated into an extra tube of culture media and allowed to incubate in the anaerobic cabinet (85% N_2, 10% H_2 and 5% CO_2, 36 °C) to ensure the strains were still able to grow under optimal conditions. A negative control tube of serum supplemented OTEB only was also set up for each

temperature investigated. For each of the six temperatures, the three newly inoculated replicate cultures of each strain along with the negative control tube were placed into an anaerobic jar (Oxoid™ AnaeroJar™ 2.5L; Oxoid Ltd, Basingstoke, UK) with an anaerobic gas generating sachet (Oxoid™ AnaeroGen™ 2.5L Sachet; Oxoid Ltd, Basingstoke, UK). The cultures were then incubated under anaerobic conditions at either 4 °C, 12 °C, 20 °C, 37 °C, 45 °C or 60 °C. Cultures were sampled on day 0, 2, 4, 7, 10, 15 and 21, except for cultures kept at 60 °C which were only sampled until day 7.

On sampling days, each of the cultures were growth and motility scored (see Table 6.1 for scoring descriptions) by phase contrast microscopy and sub-cultured into secondary tubes of fresh OTEB 10% (v/v) FCS or RS depending upon DD treponeme phylogroup. The secondary cultures were transferred into an anaerobic cabinet (85% N_2, 10% H_2 and 5% CO_2, 36 °C) for incubation. Growth and motility scores of secondary cultures were carried out using phase contrast microscopy after 7 days incubation and again at 14 days incubation if growth was poor after the initial 7 days.

6.2.5 DD treponeme cultivation under different pHs

A microplate methodology was adapted from a study investigating the minimum inhibitory concentrations (MICs) of antibiotics against DD treponemes (Evans et al., 2009a) in order to investigate DD treponeme survival in pHs 4.5-9 (0.5 increments). Hayley Crosby-Durrani, a PhD student at the University of Liverpool, aided in the completion of laboratory work for this section.

The pH of OTEB (Chapter 2 Table 2.1) used for culturing DD treponemes is normally at pH 7 ± 0.2. The pH of the OTEB 10% (v/v) RS for the *T. medium* DD treponeme phylogroup strains and OTEB 10% (v/v) FCS for the *T. phagedenis* DD treponeme phylogroup strains and the *T. pedis* DD treponeme phylogroup strains was altered by supplementing the media with a final working concentration of 100 mM of particular buffers to reach desired final pHs. These buffers are listed in Table 6.2. Given treponemes are cultivated in a commercially available complex media (OTEB), the pHs of the buffers used to alter OTEB pHs were not the same as the desired pHs due to interaction with the OTEBs own buffering system. The altered pH OTEB 10% (v/v) FCS or RS as well as the sterile 96-well polystyrene flat-bottomed microplates (Appleton Woods, Birmingham, UK) were incubated in anaerobic conditions (85% N_2, 10% H_2 and 5% CO_2, 36 °C) for at least 5 hours prior to the survival

study commencing with lids left slightly ajar to enable gas exchange. The pH of the media was measured, using a pH metre, after that time and used as the final pH.

Cultures of *T. medium* DD treponeme phylogroup strains T19 and T56, *T. phagedenis* DD treponeme phylogroup strains T320A and T354B and *T. pedis* DD treponeme phylogroup strains T3552 Band T354A were prepared as previously described in section 6.3.4.

Microplates for each DD treponeme phylogroup were set up as follows: 150 µl of each pH altered OTEB 10% (v/v) FCS or RS was added to each well in a single column with two columns of wells containing normal OTEB 10% (v/v) FCS or RS (positive controls), 50 µl of each strain were then added to each well across three rows resulting in six rows containing each DD treponeme strain (final volume 200 µl) and the two rows of wells as the bottom without bacteria act as controls for the media and were made up to a volume of 200 µl. Inoculated microplates were incubated in an anaerobic cabinet (85% N_2, 10% H_2 and 5% CO_2, 36 °C) between absorbance measurements.

Absorbance at 540nm was measured using a Multiskan microtitre plate reader (Thermo Scientific, Hampshire, UK) on day 0 and day 4 for strains from the *T. phagedenis* and *T. pedis* DD treponeme phylogroups and day 5 for *T. medium* DD treponeme phylogroup strains. To determine which pH values inhibited growth, the absorbance measurement for day 4/5 of incubation (during the late exponential phase/ early stationary phase) was compared to the measurement for day 0 (immediately after inoculation). For each DD treponeme phylogroup, the experiment was carried out in triplicate on three separate occasions and each experiment had three technical replicates of each strain. The technical replicates were averaged for each experiment and the difference in growth between the DD treponemes in the test pH and the DD treponemes in the unaltered control was compared and expressed as a decimal fraction. The average of the three separate experiments was taken as the final reported result for each DD treponeme phylogroup strain.

To determine if the pHs were bactericidal for the DD treponemes, new sterile 96-well polystyrene flat-bottomed microplates were set up for each DD treponeme phylogroup with all wells containing 180 µl of OTEB 10% (v/v) FCS or RS (pH unaltered) depending upon phylogroup. Both the microplates and media had been allowed to incubate as previously described. Following inhibitory pH determination, 20 µl of culture from each well of the different pHs from the original microplates for each strain were sub-cultured into the newly

prepared microplates resulting in a total volume of 200 µl per well. The microplates were then incubated for 5 days before growth was determined by phase contrast microscopy, with bactericidal pHs defined as pHs which contained no treponemes when sub-cultured. Sub-culturing was carried out for each of the three technical replicates and each of the three experiments carried out on different occasions for each DD treponeme phylogroup and the median of these experiments for each phylogroup was reported as the final result.

Table 6.2: Buffers used to alter media pH

Final OTEB pH	Buffer (100 mM working concentration)	Buffer pH
4.5	Sodium acetate	4.3
5	Sodium acetate	4.8
5.5	Sodium acetate	5
6	Bis-Tris	5.8
6.5	Bis-Tris	6.5
7	Bis-Tris	7.5
7.5	TAPS	8.3
8	TAPS	8.8
8.5	TAPS	9
9	TAPS	10.3

6.3 Results

6.3.1 The survival of DD treponemes cultured under different pH conditions

The growth and survival of DD treponemes at different pHs was investigated by culturing two strains from each of the three cultivable DD treponeme phylogroups (*T. medium, T. phagedenis* and *T. pedis*) in OTEB (supplemented with serum) altered to pHs of 4, 4.5, 5, 5.5, 6.0, 6.5, 7, 7.5, 8, 8.5 and 9. The pH values that are enhancing or inhibitory to growth were initially determined, followed by determination of viability. Viability was determined by subsequent inoculation into secondary media as has previously been successfully employed for determination of DD treponeme minimal bactericidal concentrations (MBCs) (Evans et al., 2009a). Results are shown in Figure 6.2, 6.3 and Table 6.3.

6.3.1.1 Determination of pHs enhancing or inhibitory for DD treponeme growth in culture

The DD treponemes were inoculated into media (within microplates) at different pH values (pHs 4-9 in 0.5 increments) alongside parallel inoculated DD treponeme strains grown under normal culturing conditions (positive control) with the latter using the inherent pH of the routinely used fastidious anaerobic bacteria growth medium: OTEB (pH 7 ± 0.2 at 25°C). After the respective incubation period the bacterial growth was measured with a microplate spectrometer (plate reader) using an absorbance of 540nm. The final growth measurement was calculated by subtracting the OD value of the respective media without treponemes inoculated (media only control wells) from each well containing treponemes for the respective media. Differences in growth were analysed between treponemes cultured at the different pH values and the positive control used for each strain to determine whether the test pH was optimum or inhibitory to growth. The growth value was taken as fold-change compared to the control (=growth at pH value/growth of control) therefore a value of 1 (or 100%) indicates that growth of the DD treponemes in the test pH was the same as the growth of the DD treponemes under normal control conditions. Values > 1 indicates growth rates at these pH values were enhanced compared to growth of the positive control and values < 1 suggest growth was less than in the positive control wells indicating inhibition. A cut off of ≥ 0.1 was used to determine if growth had occurred and a cut off of ≥ 0.75 selected for good growth.

From analysis, one or more acidic pH cultures were omitted due to precipitation occurring in the OTEB supplemented with serum which confounded results. For the *T. medium* DD treponeme phylogroup this was pH 4-5 whereas for the *T. phagedenis* and *T. pedis* DD treponeme phylogroups this was pHs 4 and 4.5.

The two strains analysed under the range of pHs for the *T. medium* DD treponeme phylogroup were T19 and T56, results are depicted in Figure 6.2a. Growth occurred for both strains between the pHs of 5.5 and 9.0 although growth at pH 5.5, 6 and 9 were considerably poorer than that of the positive control (< 0.75). The optimum pH for growth, as observed by the largest peak in the graph, for both strains was pH 7.5 with a value of 1.44 (144%), thus suggesting greater growth levels than the positive control. Both strains demonstrated a good growth range of pH 6.5 to 8.5 although growth profiles differed slightly in that T19 (but not T56) had a second peak at pH 8.5 of high growth which whilst not as high as the first peak (7.5) was still greater than the positive control. The standard error of the mean (SEM) (Figure 6.2a) showed that replicate growth at some pHs

demonstrated large spread about the mean particularly at some of the pHs where good growth was exhibited.

There were greater differences in growth observed between *T. phagedenis* DD treponeme phylogroups strains T320A and T354B than observed between the *T. medium* DD treponeme phylogroup strains. The optimum pH was pH 7 for T320A and pH 6.5 for T354B with good growth ranges of pHs 6-7 and 6.5-7 respectively (Figure 6.2b). Although pH 7 was determined to be the optimum pH for T320A there was a plateau between pHs 6 and 7 with only a gradual increase in growth between pHs 6, 6.5 and 7. However, growth rapidly dropped for pH 7.5. Whereas T354B demonstrated no plateau, and instead there was a sharp increase in growth between pH 6 and pH 6.5 (peak growth), with a gradual decline in growth in more alkali pHs following the optimum pH. As for *T. medium* phylogroup strain T19, an increase in growth was observed again at pH 8.5 following a decrease at pH 8, although for the *T. phagedenis* DD treponeme phylogroup this increase was less marked than for T19. No growth was observed for pH 5 for both *T. phagedenis* strains whilst pH 5.5 inhibited T354B only. The SEM bars were relatively small for each of the pH values except for pH 8, 7.5, 5.5 and 5 (Figure 6.2b).

Interestingly, only *T. phagedenis* DD treponeme phylogroup strain T320A exhibited growth similar to the positive control, with pH values 6.5 and 7.0 showing a marginal increase in growth compared to control with differences of 1.03 (103%) and 1.06 (106%). Interestingly, no fold change values for T354B at any pH value (including pH 7) reached 1 suggesting that all pH conditions resulted in growth with some level of inhibition.

The two strains for the *T. pedis* DD treponeme phylogroup showed less differences in growth at different pHs than was observed the *T. phagedenis* DD treponeme phylogroup, although growth of *T. pedis* strain T354A was slightly reduced for each pH when compared to *T. pedis* DD strain T3552B (Figure 6.2c). For both strains, the optimum pH value for growth was pH 7.5, although as previously observed for *T. medium* phylogroup, a second growth peak appeared at pH 8.5 (with a prior a decline at pH 8) where growth was only marginally less than that of the optimum pH. The *T. pedis* DD treponeme phylogroup demonstrated a large pH range enhancing growth, rivalling that of the *T. medium* DD treponeme phylogroup, with substantial growth observed between pH 6.5 and 9. Interestingly, for the majority of the pH values in which substantial growth was observed, growth was enhanced (value > 1) at these pH values compared with the positive controls. Only pH 9 for both strains showed reduced growth compared to positive controls within

this growth enhancing pH range. No growth was observed for pH 5 and 5.5 for both strains as well as pH 6 for strain T354A. Generally the standard error bars were small for the *T. pedis* DD treponeme phylogroup, particularly for T354A in which error bars were negligible for some pHs. For both strains there was a larger spread of growth about the mean for the replicates at either end of the pH range and T3552B demonstrated a larger spread about the mean for pH 7.5-8.5 as well.

6.3.1.2 Determination of viability of DD treponemes following incubation in culture at a range of pH values

The six strains representative of the three DD treponeme phylogroups (*T. medium, T. phagedenis* and *T. pedis*) cultured at a range of pH values in the previous section were sub-cultured into OTEB (supplemented with 10% (v/v) the respective serum) with the media unaltered in pH (inherently at a pH 7 ± 0.2 at 25°C) to determine if the DD treponemes were viable following exposure to the pH range investigated. Growth was scored as described in Table 6.1 and the results summarised in Table 6.3. A growth score of two or above was considered viable.

Of particular interest were the pH values which appear to be fully or partially inhibitory of treponeme growth. For the *T. medium* DD treponeme phylogroup strains, growth was low at pH 5.5, 6 and 9 in the initial experiment. T19 appeared to be viable at all three of these pH values with pH 9 obtaining a median growth score of 3 when sub-cultured, similar to pH values for which substantial growth was achieved. On the other hand T56 achieved a median growth score of two for pH 6 and 9 in the secondary media but did not appear to be viable at pH 5.5.

With regards to the *T. phagedenis* DD treponeme phylogroup strains, as aforementioned both were inhibited at pH 5 and 5.5 whilst at pH 6 only T354B was inhibited with no growth observed in initial cultures. Following sub-culturing these DD treponemes originally cultured at pH 5 were not viable for either strain whilst at pH 5.5 only T354B was not viable. In contrast, sub-cultures of T320A from pH 5.5 and T354B from pH 6 showed substantial growth with median growth scores of four and three respectively in the secondary culture. Interestingly, whilst poor growth compared to the positive control was observed for pHs greater than 7 for both *T. phagedenis* phylogroup strains, similar growth scores were achieved in secondary media to the strains when exposed to pH values that were considered to have good growth in the original cultures.

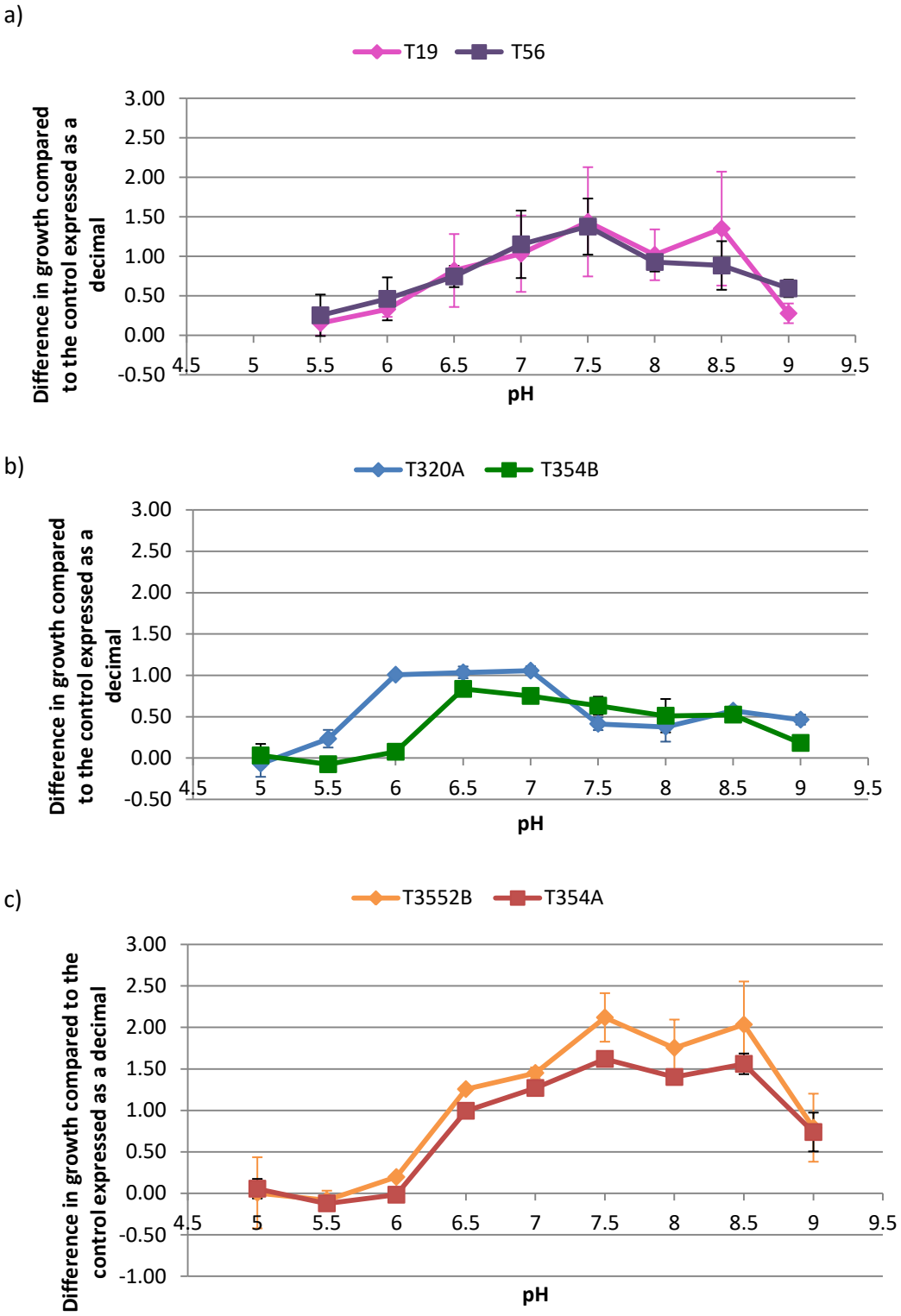


Figure 6.2: Mean growth of DD treponemes cultured in different pHs

a) T. medium DD treponeme phylogroup strains T19 and T56 mean growth difference compared to the respective strains controls for each pH. b) T. phagedenis DD treponeme phylogroup strains T320A and T354B mean growth difference compared to the respective strains controls for each pH. c) T. pedis DD treponeme phylogroups strains T3552B and T354A mean growth difference compared to the respective strains controls for each pH. Each DD treponeme phylogroup strain was cultured in each pH in triplicate and on three different occasions with the mean results shown here. The error bars in each figure represent ± standard error of the mean (± SEM).

Table 6.3: Growth scores of DD treponemes sub-cultures previously exposed to different pHs
Median growth scores range from 0-5 with 0 being no growth and 5 being dense growth (see Table 6.1).

DD treponeme phylogroup	Strain	pH								
		5	**5.5**	**6**	**6.5**	**7**	**7.5**	**8**	**8.5**	**9**
	T19		2	2	3	3	3	3	3	3
T. medium	**T56**		1	2	2	2	3	3	3	2
	T320A	1	4	4	4	3	3	4	3	3
T. phagedenis	**T354B**	0	0	3	3	3	3	3	3	3
	T3552B	0	1	2	3	3	3	3	3	3
T. pedis	**T354A**	0	0	3	3	3	3	3	3	3

Similarly for *T. pedis* DD treponeme phylogroup strains, pH 5 and 5.5 were inhibitory for both strains growth and pH 6 was also inhibitory for T354A in the original cultures. In secondary culture both strains appeared to not be viable when previously exposed to pH 5 and 5.5. However, T354A achieved a median growth score of three in secondary culture following exposure to pH 6. Poor growth had been observed for T3552B at pH 6 but the treponemes were still viable with a median score of two in secondary media.

For all three DD treponeme phylogroups pH 6-9 did not show bactericidal activity. Similar median growth scores, when sub-cultured, for each of these pH values were achieved despite differences in initial growth levels. For example, in the original culture for the *T. pedis* DD treponeme phylogroup strain T3552B, differences in growth to the control of 2.12 (212%) and 0.79 (79%) were obtained for pH 7.5 and 9 respectively. However, in secondary culture median growth scores of three were obtained for T3552B from both pH values. Interestingly, a median growth score of four was achieved in secondary cultures for *T. phagedenis* phylogroup strain T320A, for pH 6 and pH 5.5 despite having had very different levels of initial growth with 0.23 (23%) and 1.01 (101%) fold change respectively when compared to the positive T320A control.

6.3.2 DD treponeme growth and survival at a range of temperatures

A single strain from each of the *T. medium, T. phagedenis* and *T. pedis* DD treponeme phylogroups was inoculated into OTEB (supplemented with 10% (v/v) of respective serum) and incubated anaerobically at a temperature of either 4 °C, 12 °C, 20 °C, 37 °C, 45 °C or 60 °C for a total of 21 days (except for 60°C which was 7 days) to determine growth and

survival at these temperatures. Viability following incubation at these temperatures was determined by sub-culturing into secondary media on days 0, 2, 4, 7, 10, 15 and 21. Growth and motility was scored for each culture and a growth score of two or more was considered as growth. The length of survival was determined as the last day a score of two (or more) was obtained (median of triplicate results). Results are summarised in Table 6.4. Growth and motility score tables are in appendix C.

6.3.2.1 Direct observation of DD treponemes in initial cultures incubated at various temperatures anaerobically

All DD treponeme phylogroup strains (*T. medium* phylogroup strain T19, *T. phagedenis* phylogroup strain T320A and *T. pedis* phylogroup strain T3552B) tested were present the full duration of the study (21 days for all temperatures except 60 °C which was 7 days) when incubated at temperatures of 4 °C, 12 °C, 37 °C, 45 °C and 60 °C (Table 6.4). Whilst *T. phagedenis* phylogroup strain T320A and *T. pedis* strain T3552B were present the full duration at 20 °C, *T. medium* phylogroup strain T19 was only present until the 10 day time point. Additionally, *T. medium* phylogroup strain T19 only was only present for 4 days at 60°C.

Growth scores were generally low (scores of two to three) in all microcosms except the 37°C microcosm which reached median growth scores of four and five (see appendix C). These growth scores stayed relatively steady throughout the time points, rarely changing by more than one (except for 37°C which saw an increase of two following the first time point after inoculation). Interestingly, there did appear to be a sustained small increase in growth (from growth score two to three) at 4°C between days 7 and 10, and 7 and 15 for T320A and T3552B respectively before declining again. With regards to motility, there appeared an overall trend for motility to decrease with time irrespective of growth score.

6.3.2.2 Viability of DD treponemes in secondary culture incubated at 37 °C

Sub-culturing of *T. medium* DD treponeme phylogroup strain T19 at 37 °C for 7 days, from cultures incubated at the various temperatures revealed that this strain remained viable for the full 21 days following incubation at 4 and 37°C (Table 6.4). However, T19 only remained viable for 15 days at 12 °C, 7 days at 20°C and could only be detected following initial inoculation at temperatures of 45 and 60 °C and not subsequently.

Sub-culturing of *T. phagedenis* DD treponeme phylogroup strain T320A and *T. pedis* DD treponeme phylogroup strain T3552B showed similar patterns of viability following incubation at various temperatures. Both phylogroup strains remained viable for the full duration of incubation at 12 °C, 20 °C and 37 °C and were not viable when incubated at 60 °C, except for the initial inoculation on day 0, demonstrating treponemes were viable upon initial inoculation. However, T3552B only remained viable for 15 days at 4 °C whereas T320A was viable at this temperature for the full duration of the study. Additionally, at 45 °C, T320A remained viable up to the 2 day time point whereas T3552B did not. Sub-cultures were checked again at 2 weeks where growth did not seem viable and in no cases was growth seen.

Table 6.4: Maximum number of days DD treponemes observed and remain viable at different temperatures[a]

Temperature[b]	Strain no.[c]	Median No. days DD treponeme presence in Microcosm	No. days survival as determined by sub-culture	
			Sub-culture median	Sub-culture range
4°C	T19	≥ 21	≥ 21	15 - ≥ 21
	T320A	≥ 21	≥ 21	≥ 21
	T3552B	≥ 21	15	15
12°C	T19	≥ 21	15	10 - ≥ 21
	T320A	≥ 21	≥ 21	≥ 21
	T3552B	≥ 21	≥ 21	≥ 21
20°C	T19	10	7	7
	T320A	≥ 21	≥ 21	≥ 21
	T3552B	≥ 21	≥ 21	≥ 21
37°C	T19	≥ 21	≥ 21	≥ 21
	T320A	≥ 21	≥ 21	≥ 21
	T3552B	≥ 21	≥ 21	≥21
45°C	T19	≥ 21	0	0
	T320A	≥ 21	2	2
	T3552B	≥ 21	0	0
60°C	T19	4	0	< 0 - 0
	T320A	7	0	0
	T3552B	7	0	0

[a] *Number of days observed and survival is based on growth scores.*

[b] *Incubation at each temperature was carried out for 21 days except for 60 °C which was only 7 days.*

[c] *T19 belongs to the T. medium DD treponeme phylogroup, T320A belong to the T. phagedenis DD treponeme phylogroup and T3552B belong to the T. pedis DD treponeme phylogroup.*

Growth scores were high following sub-culturing for 7 days in 37 °C after exposure to the different temperatures with the vast majority of time points scored as either four or five. Only on two occasions were growth scores lower than this. Following exposure of T3552B to 4 °C for 21 days the median growth score of the secondary culture for this time point was one. A median growth score of three was obtained after 7 days for secondary cultures of T19 which had been exposed to 12 °C for 15 days. T19 could not be visualised in secondary media on the next (21 day) time point for 12 °C.

Motility following exposure to each of the temperatures was low in secondary cultures for T3552B with median motility scores of predominantly two or below. On the other hand, T19 and T320A mainly had median motility scores between three and five in secondary cultures following previous exposure to the different temperatures. An exception to these high motility scores for T19 was secondary cultures from the 37 °C microcosms which mainly had median motility scores of two from day 2 onwards. Interestingly, the secondary culture of the day 2 time point at 45 °C for T320A (maximum survival at this temperature) had a median motility score of five which was higher than the maximum median score of four achieved for 37 °C secondary cultures.

6.3.3 Direct observation and survival of DD treponemes in faecal microcosms incubated aerobically at 12 °C

Bovine faecal microcosms were inoculated with either *T. medium* DD treponeme phylogroup strain T19, *T. phagedenis* DD treponeme phylogroup strain T320A or *T. pedis* DD treponeme phylogroup strain T3552B and incubated aerobically at 12 °C for 7 days to determine DD treponeme survival and growth in bovine faeces. On each day of incubation, a sample of the microcosm was sub-cultured in OTEB supplemented 10% (v/v) the respective serum (Chapter 2 Table 2.1) and incubated anaerobically at 37 °C for 7 days (cultures were incubated for 28 days if growth was poor) to determine whether the treponemes in the microcosm were still viable. Survival and viability was interpreted as stated in section 6.3.2. Results are summarised in Table 6.5 and median growth and motility scores can be found in appendix C.

6.3.3.1 Direct microscopic observation of DD treponemes within bovine faecal microcosms incubated aerobically at 12 °C

Each strain from the three DD treponeme phylogroups investigated could be observed for the full 7 days within the faecal microcosms using direct phase contrast microscopy (Table

6.5). The median growth score did not vary between time points for each strain and was the same as the growth scores obtained in each strains control microcosm. For all strains, the median motility scores obtained for each time point in the faecal microcosms was one. This was also true for the T19 control microcosm. However, for the T320A and T3522B control microcosms, median motility scores were initially higher than one with T320A obtaining median motility scores between two and three until day 3 and T3552B obtaining a median motility score of two on days 0 and 1.

6.3.3.2 Viability of DD treponemes from faecal microcosms as determined by sub-culture at 36 °C in anaerobic conditions

T. medium DD treponeme strain T19 was identified as not viable following incubation in the faecal microcosm at 12 °C, with growth only occurring in the secondary culture when sampled immediately after inoculation (day 0) into the faecal microcosm (Table 6.5). Similarly, the T19 control incubated in the same conditions in a microcosm which contained only the respective growth media (OTEB supplemented with 10% (v/v) RS) was also not viable following inoculation into secondary media except for in the secondary culture taken shortly after inoculation into the microcosm (day 0). Median growth scores for secondary media from day 0 in the microcosms were three and two for T19 in the faecal microcosm and control microcosm respectively, reaching a median score of four for both after 28 days incubation. Thus T19 was viable upon initial inoculation. The median motility score was three for both T19 and the control on day 0.

Table 6.5: Maximum number of days DD treponemes observed and remain viable in bovine faecal microcosms[a]

Phylogroup	Strain no.	Median No. days DD treponeme presence in Microcosm	No. days survival as determined by subculture	
			Week 1 median	Week 1 range
T. medium	**T19**	≥ 7	0	≤ 0
	T19 Control	≥ 7	0	≤ 0
T. phagedenis	**T320A**	≥ 7	1	1 - 3
	T320A Control	≥ 7	6	6 -≥ 7
T. pedis	**T3552B**	≥ 7	1	0 -6
	T3552B Control	≥ 7	6	1 - ≥ 7

[a] Number of days observed and viable are based on growth scores. Incubation was for a maximum of 7 days in microcosms.

Contrastingly, both *T. phagedenis* DD phylogroup strain T320A and *T. pedis* DD phylogroup strain T3552B were viable in the faecal microcosms, as determined by sub-culturing, for up to 1 day with a range of 1-3 days for T320A and 0-6 days for T3552B. Whereas T320A and T3552B in the control microcosms were viable for a median of 6 days with T3552B ranging between 0 and 6 days and T320A identified with a narrow range due to consistent viability of 6 and 7 days. Where T320A from the faecal and control microcosms were viable in secondary media, growth and motility was high with median growth scores of four and five and median motility scores between two and four. However, growth decreased down to median scores of two and one for the T320A control in secondary media when inoculated from the control microcosm on days 6 and 7 respectively. On the other hand, T3552B median growth and motility scores in sub-culture were initially substantial with median growth scores of four and five and median motility scores of three on day 0 for T3552B faecal microcosm sub-culture and on days 0-1 for the control sub-culture. However, growth and motility subsequently declined to a median growth score of two and median motility score of one until on day 2 the faeces sub-culture was not viable and day 7 the control was no longer viable.

6.3.4 Direct observation and survival of DD treponemes in five different bedding microcosms incubated aerobically at 12 °C

Strain T320A, belonging to the *T. phagedenis* DD treponeme phylogroup, was inoculated into microcosms of five different bedding types (straw, sawdust, RMS, sand and a sand and 5% lime mix) and incubated aerobically at 12 °C for 7 days to determine the ability of DD treponemes to survive in different types of bedding commonly used in dairy systems. Viability was measured by sub-culturing samples of the microcosms on each of the 7 days of the experiment into OTEB supplemented with 10% (v/v) with the respective serum and allowed to incubate anaerobically at 37 °C for 7 days. Direct observation of material from within microcosms together with viability as determined by sub-culture was interpreted as in section 6.3.2. Results are summarised in Table 6.6. Median growth and motility score tables are in appendix C.

6.3.4.1 Direct observation of DD treponemes within five different bedding microcosms incubated aerobically at 12 °C

The *T. phagedenis* DD phylogroup strain T320A was present, as determined by direct microscopic observation, for the full 7 days in microcosms consisting of sawdust, RMS and

sand (Table 6.6). In the straw microcosm, T320A could be visualised on day 0 shortly after inoculation but not on the days following. Interestingly, T320A could not be observed and therefore was considered physically destroyed in the sand 5% (w/w) lime mix shortly after inoculation on day 0.

Sand and the control microcosms consistently had a median growth score for the test DD treponeme of four throughout the incubation period. For both sawdust and RMS a median growth score of four was given on day 0 which lowered to three for days 1-6 and became two on day 7. A low median growth score of two was observed for straw on day 0. Motility was scored as one in all microcosms except for the control in which median scores of two were also observed. Interestingly, when observing the sand microcosm, a phenomenon in which the sand appeared to 'stick' to the DD treponemes was observed (Figure 6.3). Figure 6.3a shows what the negative control sand microcosm looks like under the microscope with no DD treponemes present whereas Figure 6.3b shows a sand microcosm containing DD treponemes in which clear treponeme shaped outlines can be observed in the sand.

Table 6.6: Maximum number of days DD treponeme observed and remain viable in different types of bedding microcosms

Number of days observed and viable are based on growth scores. (–) denotes no survival. Incubation was for a maximum of 7 days in microcosms.

	1. No. days DD treponeme presence[1]		No. days survival as determined by sub-culture	
Bedding	**Microcosm median**	**Microcosm range**	**Sub-culture median**	**Sub-culture range**
Straw	0	0 - 3	-	-
Sawdust	≥ 7	≥ 7	6	6
RMS	≥ 7	≥ 7	5	4 - 5
Sand	≥ 7	≥ 7	≥ 7	≥ 7
Sand 5% lime	-	-	-	-
Control	≥ 7	≥ 7	≥ 7	≥ 7

[1] *Data for T. phagedenis DD treponeme phylogroup strain T320A.*

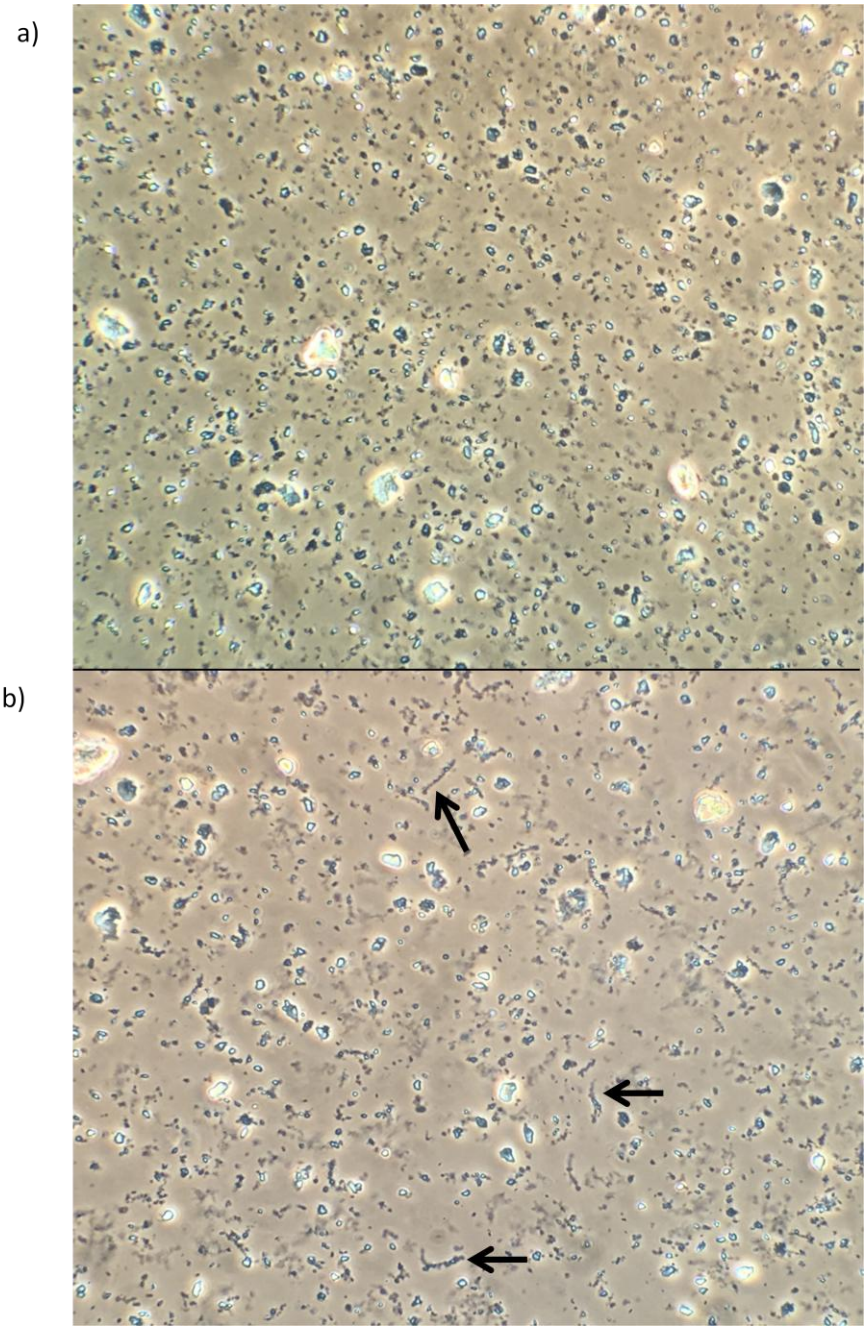


Figure 6.3: Sand microcosms

a) Negative control microcosm with sand particles containing no DD treponemes. b) Sand microcosm containing DD treponemes (of which arrows show examples of) to which the sand particles appears to be stuck.

6.3.4.2 Viability of DD treponemes in bedding microcosms as determined by sub-culture at 36 °C in anaerobic conditions

Following sub-culture, viability of *T. phagedenis* DD treponeme T320A was determined for each of the bedding microcosms. T320A remained viable in sand for the full 7 day duration of incubation in the microcosm (Table 6.6). In sawdust, T320A remained viable for 6 days and in RMS remained viable for a median of 5 days (4-5 day range between replicate experiments). On the other hand, T320A was not at all viable following incubation, however short, in the straw or sand 5% (w/w) lime microcosms. The T320A control microcosm which only contained T320A in OTEB 10% (v/v) FCS remained viable throughout the incubation period.

Median growth scores for sub-cultures (after 7 days incubation) from sand stayed consistently at three for each time point. Sawdust on the other hand had a median growth score of two in sub-cultures from each time point except the final time point which was a median score of one. RMS sub-cultures started with a median growth score of three and then declined to a median growth score of two until day 5. For the T320A control when sub-cultured growth scores gradually decreased over the 7 days from a median score of five to two. In microcosms which had either no growth or poor growth in sub-culture after 7 days did not change after 28 days incubation.

Motility was low in all secondary cultures from bedding microcosms, mainly staying at a median motility score of one. However, RMS and sand did initially, on day 0, have higher median motility scores of four and two respectively. On the other hand, the control microcosm had high median motility scores of four and three in early time point subcultures which gradually declined to less than one by day 7.

6.4 Discussion

This study aimed to understand the ability of DD treponemes to grow and remain viable under varying conditions that may be found on farm or in the host (i.e. cattle, sheep etc) to provide further insight into the viability of identified infection reservoirs for transmission as well as providing knowledge that could help determine or discredit newly proposed infection reservoir sites for DD. In particular, the study investigated generic conditions of pH and temperature which are important not only in terms of infection reservoirs but for culturing conditions used frequently as a method of detecting DD treponemes in reservoir environments. Furthermore, two specific niches, bovine faeces and bedding commonly

used on dairy systems, were investigated to determine their role in treponemal survival and possible transmission on farm.

There is a dearth of information on the effect of different pHs on DD treponeme growth and viability. This study demonstrated that DD treponemes phylogroups have slight variances in their preferred pH optimums and ranges for growth. The *T. medium* DD phylogroup preferred growth between the pHs of 6.5-8.5 with an optimum pH for growth of pH 7.5 for both strains investigated. The *T. pedis* DD phylogroup was similar in that its optimum pH was 7.5 although it had a slightly wider pH range of 6.5-9. The *T. phagedenis* DD treponeme phylogroup on the other hand had slightly lower optimum pHs, with strain T320A preferring pH 7 (6-7) and strain T354B having an optimum pH of 6.5 (6.5-7). This is in contrast to the aforementioned previous study (Section 6.1) which found the optimum pH for an isolate belonging to the *T. phagedenis* DD treponeme phylogroup to be pH 7.4, with a wider pH range for growth of 6.5-8 (Wilson-Welder et al., 2013). Furthermore, Bergey's manual states that the species *T. phagedenis* in general does not grow at pH 6 (Brenner et al., 1984), and although growth did not occur for one strain at this pH, the other strain grew just as well at pH 6 as it did in the control well with no pH alterations. Thus it would appear for the *T. phagedenis* DD treponeme phylogroup at least, there may be within phylogroup differences in pH tolerance. Investigations using a larger number of strains per phylogroup would help to delineate these differences. However, it must be noted that different culture media and methods of pH adjustment were used in different studies and thus other factors may have had an effect on growth and viability between studies as well as pH (Brenner et al., 1984; Wilson-Welder et al., 2013).

In this thesis, the media used for the detection and maintenance of DD treponemes has an inherent pH of 7 (± 0.2 at 25°C). Thus whilst all three DD treponeme phylogroups are able to grow well at this pH it may bias for treponemes of the *T. phagedenis* DD treponeme phylogroup, similar to strain T320A, which display optimum growth at pH 7. Indeed, *T. phagedenis* DD treponemes are the most widely isolated of the DD treponemes (Evans et al., 2008; Yano et al., 2009). Where it may be suspected that the *T. pedis* or *T. medium* phylogroup is present in a mixed culture it may be worth investigating whether altering the pH towards pH 7.5 will increase the likelihood of these phylogroups being isolated due to more optimum conditions leading to increased levels of growth over other bacteria. Furthermore this may change the time required for these phylogroups to reach late

exponential/ stationary phase and thus growth curves in these conditions may warrant exploration.

Interestingly, for the *T. pedis* and *T. medium* DD treponeme phylogroups growth was enhanced (greater than 100%) for a few of the mid-range pHs (e.g. 6.5-8.5 for *T. pedis* phylogroup) when growth was compared to the positive control for each strain, which only contained OTEB (pH 7 ± 0.2 at 25 °C) and serum normally used for treponeme culture. Whilst this may mainly be the result of a better pH for growth for some of the test pHs, the enhanced growth in the test pH 7 (same as the unaltered OTEB control) suggests that adding the Bis-Tris buffer to the OTEB may have also had an effect on improving growth. This enhancing effect was observed at pH 7 for *T. medium* phylogroup strain T56 and both strains of the *T. pedis* phylogroup. It may be that by adding the buffer to the OTEB (supplemented with the respective serum) it diluted the media slightly which may have been beneficial for growth for those phylogroup strains or it may be that the buffer contained a chemical or property advantageous to the growth of these strains. As the exact same level of growth compared to the control did not occur for all test pHs obtained using this buffer for these strains then it is highly likely that pH was also having an effect on growth. However, the *T. phagedenis* DD treponeme phylogroup did not show enhanced growth at any pH; although for T320A pH 7, growth was very close to that of the control. For *T. phagedenis* phylogroup strain T354B, growth was actually reduced compared to the control for pH 6.5 (the optimum test pH for this strain) and pH 7. Thus the buffer may have the opposite effect on this strain and a similar observation of the buffer itself affecting growth has been made in a *Leptospira* spp. pH survival study (Parker and Walker, 2011) .

Knowing the pH range that DD treponemes can survive in is important for assessing whether a particular site could support DD treponeme survival and thus act as a reservoir for infection. For example the skin surface of the bulb of the heel near to where DD lesions form in cattle has been cited as having a pH of 7.1 (range of 6.5-7.5), thus any of the three DD treponeme phylogroups would be able to survive their based on pH alone (Meyer and Neurand, 1991).

The rumen, in which DD treponemes have previously been identified in the fluid (Nascimento et al., 2015; Zinicola et al., 2015b), usually has a pH between 6 and 7 which would enable survival of DD treponemes (Krause and Oetzel, 2005; Kleen et al., 2013). However, sub-acute ruminal acidosis (SARA) can result in a drop in rumen pH below 6 for periods of time and often occurs in high yielding dairy cattle fed high grain diets (Krause

and Oetzel, 2005; Kleen et al., 2013). The knowledge that DD treponemes do not seem to survive in acidic pHs (for example *T. phagedenis* DD treponeme phylogroup strain T354B did not survive in pHs of 5.5. or 5) implies that infection reservoirs would not be found in places with similar or lower acidic pHs and thus during periods of SARA DD treponeme remaining viable in rumen fluid is unlikely. Additionally, hindgut acidosis was postulated to be associated with DD treponeme presence in the RAJ and faeces due to the damage it causes to the epithelium lining and the shedding of mucin casts (see Chapter 4 Section 4.1). However, hindgut acidosis reduces the pH in the hindgut digesta, which would be unfavourable to DD treponemes if they were in contact with the lumen and reduces the pH of faeces to below 6 which would also negatively affect DD treponeme viability in faeces (Gressley et al., 2011). Furthermore, whilst it may be expected that the lack of oxygen during fermentation of forage to produce silage would produce a good anaerobic environment for DD treponeme survival, fermentation lowers the pH, resulting in silage with a pH between 3 and 5 in which DD treponemes could not survive (Danner et al., 2003).

Another factor which affects whether DD treponemes may be able to survive in a particular reservoir is temperature. It is known from culturing that DD treponemes are able to survive and grow between 36 °C and 37 °C (Evans et al., 2008). Further investigation into temperatures in which DD treponemes survive found survival was possible at temperatures of 4 °C, 12 °C, 20 °C and 37 °C for all three DD treponeme phylogroups, although the *T. medium* DD treponeme phylogroup survived for a shorter period of time at 12 and 20 °C than the other two DD treponeme phylogroups. Visible growth at these temperatures only occurred at 37°C. However, at the lower temperatures the treponemes remained viable and were able to grow again once sub-cultured at 36 °C. These results are in agreement with another study which found temperatures lower than 29 °C resulted in bacteriostatic effect on growth but the *T. phagedenis* DD treponeme strain investigated remained viable (Wilson-Welder et al., 2013). Thus temperatures down to 4 °C alone will not prevent DD treponeme survival in the environment and whilst refrigeration may prevent growth as with many other bacteria, it will not kill the DD treponemes when stored for 21 days.

Interestingly, diseases affecting the foot, including DD, raises the temperature of the hoof through inflammatory processes from its normal temperature of 21-23 °C to approximately 27-30 °C as determined by infrared thermography (Stokes et al., 2012b). The raised temperature would then enable treponemes which have infected the foot from colder

temperatures to replicate and thus be involved in disease pathology (Wilson-Welder et al., 2013).

An optimum temperature range was not investigated in this study, choosing to sample a limited number of temperatures from a wide range of temperatures instead. The core body temperature of cattle ranges narrowly around 39°C (Suthar et al., 2011) and DD treponemes have been detected within the GI tract of the host, thus it would be interesting to determine if the optimum temperature for DD treponemes is the 37 °C for which they are grown in culture at or whether the optimum is closer to body temperature of cattle. Indeed, 39 °C is within the temperature growth range for *T. phagedenis, T. vincentii* and *T. denticola* (Brenner et al., 1984) the latter two of which are closely related to *T. medium* and *T. pedis* respectively, thus it would be fair to assume this temperature would be within the range of the DD treponemes. Indeed, Wilson-Welder et al., (2013) found the optimum temperature of a DD lesion isolate belonging to the *T. phagedenis* DD treponeme phylogroup to grow optimally at 40 °C. Thus, further studies should be conducted to determine temperature range and optimum temperature for growth of each of the three DD treponeme phylogroups.

Incubation at temperatures of 45 °C and 60 °C resulted in non-viable DD treponemes, although T320A belonging to the *T. phagedenis* DD treponeme phylogroup remained viable for 2 days in secondary media at 36 °C following incubation at 45 °C. This is in concurrence with Bergey's manual for systemic bacteriology which states *T. phagedenis* either only grows slightly or not at all at 45 °C (Brenner et al., 1984). The lack of viability of DD treponemes at 45 °C and 60°C suggests that DD treponemes would not survive composting or pasteurisation techniques where temperatures can reach above 70 °C and thus composting or pasteurisation may prove effective methods of removing DD treponemes from certain environments.

Growth in media when culturing, provides treponemes with a nutrient rich stable environment in which to grow. Whilst the temperature and pH experiments using culture alone give an indication of the roles of these factors in DD treponeme survival, it is not fully representative of the complex, dynamic conditions the DD treponemes may encounter in the environment. Therefore investigation into the survival of DD treponemes in environmental matrixes were conducted.

Faeces has come under scrutiny as a potential infection reservoir of DD, in part due to the identification of DD treponemes in the GI tract and also because it is often in close contact with the skin of the foot (Evans et al., 2012b; Klitgaard et al., 2014, 2017; Nascimento et al., 2015; Zinicola et al., 2015b). Whilst conventional PCR and culture methods have not detected DD treponemes in bovine or ovine faeces (Evans et al., 2012b; Sullivan et al., 2015a), deep sequencing techniques have been able to detect DD treponeme DNA as a very small fraction of the faecal microbiota (Klitgaard et al., 2014; Zinicola et al., 2015b). The detection of DD treponemes by molecular methods in bovine faeces and the lack of detection by culture poses the question of whether DD treponemes are viable in bovine faeces. Other spirochaetes have been investigated for their survival in various animal faeces and results have varied from 210 days for *Brachyspira pilosicoli* in porcine faeces to less than 4 days for the same spirochaete in avian ceacal faeces (Boye et al., 2001; Phillips et al., 2010).

Whilst all three DD treponeme phylogroups (*T. medium, T. phagedenis* and *T. pedis*) could be visualised in the faecal microcosms for the full duration of the study with negligible change in growth, *T. medium* DD treponeme phylogroup strain T19 was not viable in bovine faeces except for the day 0 time point taken shortly after initial inoculation, however, the same results occurred for the T19 control, which was in media rather than faeces. It may therefore be deduced that aerobic incubation at 12°C contributed to the loss of viability in T19 as opposed to the faeces. In the temperature survival study *T. medium* showed a loss of viability after 15 days at 12°C in anaerobic conditions so it is highly like the oxygen concentration contributed largely to inhibiting T19 survival. The interplay of two factors for survival has been demonstrated for *Leptospira* which demonstrated a temperature biased interaction between temperature and pH upon *Leptospira* survival in culture (Parker and Walker, 2011).

On the other hand, *T. phagedenis* DD treponeme phylogroup strain T320A and *T. pedis* DD treponeme phylogroup strain T3552B had median viability of 1 day in the faeces with a range of 1-3 days viability and 0-6 days respectively. The controls for these strains both remained viable for 6 days of aerobic incubation at 12°C and thus it could be postulated that the faeces had a negative effect on *T. phagedenis* and *T. pedis* DD treponeme phylogroup viability. Interestingly, the range for the *T. pedis* DD treponeme group was larger between the replicate experiments carried out on different days; this may be accounted for by the difficult nature of cultivating DD treponemes.

Although survival for one day in faeces would appear to be short, this would still allow enough time for a cow to come into contact with the faeces containing DD treponemes and thus possibly enable transmission. Especially as the liquid consistency of dairy cattle faeces results in the hind feet becoming covered with this potentially infectious material and housed dairy cattle often spend a large amount of time with their hind feet in slurry. It is not feasible to remove faeces immediately after defecation and slurry often builds up over a short period of time in housing systems. Although regular scraping through manual and automatic means helps to control levels of slurry it never completely removes it, especially as floor design and quality may result in retention of the slurry (Wells et al., 1999; Somers et al., 2003, 2005; Frankena et al., 2009). Furthermore, automatic scraper systems and their frequency of use have actually been linked to an increased risk of DD, which may be a result of the wave of faeces carried by the scrapers coming into contact with the feet of cows slow to move out the way or the automatic scrapers may damage the feet upon impact, enabling entry of the DD treponemes from the environment (Somers et al., 2005; Cramer et al., 2009; Barker et al., 2010). Additionally, walkway and chute design often results in cattle walking directly behind each other and thus they are highly likely to step in other cows faeces shortly after defecation. Therefore, there are a variety of reasons for which cattle feet may come into contact with faeces within one day. However, this data does suggest that cows would not be at risk from DD treponemes in stored slurry.

Interestingly, *T. phagedenis* DD phylogroup strain *T320A* control and the *T. pedis* DD phylogroup strain T3552B control demonstrated the ability to remain viable under aerobic conditions at 12°C for 6 days. DD treponemes were typically considered to be strict anaerobes (Brenner et al., 1984; Stamm et al., 2002a; Evans et al., 2009a; Döpfer et al., 2012a; Wilson-Welder et al., 2015). However, there have been anecdotal reports that DD-associated treponemes may actually show levels of aerotolerance and the survival of DD treponemes on fomites also suggests a level of aerotolerance (Sullivan et al., 2014a; Angell et al., 2017). Furthermore in an unpublished preliminary study conducted by Evans et al., comparing the survival ability of T320A and T3552B when incubated in culture either aerobically or anaerobically at 36°C, it was found that both strains remained viable in aerobic conditions and demonstrated growth over the seven day period by spectrometry and phase contrast microscopy growth scoring. Further investigation into DD treponeme survival in various oxygen concentrations is required to further delineate the anaerobic nature of these spirochaetes and understand the survival and relative risk of infection from viable DD treponemes in environmental infection reservoirs exposed to oxygen.

Whilst bedding was not investigated as a potential infection reservoir for DD treponemes in this study, pathogens relating to other diseases such as the mastitis causing pathogens *Escherichia coli, Klebsiella pneumonia, Mycoplasma bovis* and *Streptococcal* spp., have been found in bedding and bedding type has been cited as a risk factor for the disease (Hogan and Smith, 1997; LeJeune and Kauffman, 2005; Godden et al., 2008; Justice-Allen et al., 2010). Healthy feet and DD lesion affected feet come into close contact with bedding and thus it is feasible that transference of DD treponemes may occur especially given that this thesis has described floor surfaces as potential fomites in Chapter 4. There is a dearth of information relating to bedding types and DD. Although the benefits of certain housing systems have alluded to straw yards having lower risk of DD and deepness of the bedding has been investigated as a risk factor (Laven, 1999; Somers et al., 2005; Barker et al., 2009), the actual risk between type of bedding material and DD prevalence has not been reported to the best of the author's knowledge. Thus the survival of DD treponemes in bedding was investigated to determine the feasibility of bedding as an infection reservoir for DD.

There were clear differences in DD treponeme survivability between the five types of bedding investigated. DD treponemes remained viable, as determined by secondary culture, in sawdust bedding for 6 days and in sand for the full 7 day duration of the study. Sand is a popular bedding choice in part as it is inert and provides poor support to pathogen growth, thus it usually has lower bacterial loads than other bedding types (LeJeune and Kauffman, 2005; Godden et al., 2008; Justice-Allen et al., 2010; Westphal et al., 2011; AHDB Dairy, 2014), resulting in its use as a method for controlling pathogens (AHDB Dairy, 2014). Therefore it is surprising that the fastidious DD treponemes survived and remained viable for 7 days within the sand microcosm given the detail above, although sand does have a pH range typically between 7 and 9 (Godden et al., 2008) which is supportive to DD treponeme survival. Sawdust, on the other hand, is known to contain higher bacterial loads particularly when wet/ soiled (Zdanowicz et al., 2004; LeJeune and Kauffman, 2005; AHDB Dairy, 2014).

RMS also known as 'green bedding' involves the recycling of cattle manure / slurry on farms using a separator designed to separate the solid fraction from the liquid fraction of manure/ slurry in order to produce RMS which is > 35% dry matter (Keys et al., 1976; Timms, 2008; Leach et al., 2015). RMS provides a cheaper alternative to other bedding types, is comfortable and is readily available (Leach et al., 2015). RMS has gained traction in many countries where it is now commonly used and is sanctioned for use in the UK,

although it is subject to strict conditions of use to try and control for risks of disease spread posed by the RMS being an animal by-product. (Leach et al., 2015; AHDB Dairy, 2016b). Research is ongoing to determine the risks of RMS use and its effect on disease prevention and control, and of particular interest is the pathogen loads in RMS which have been shown to be comparable if not higher in RMS than in other bedding types depending upon microorganism of interest (Godden et al., 2008; Timms, 2008; Leach et al., 2015; Bradley et al., 2016). In this study, *T. phagedenis* DD treponeme phylogroup strain T320A remained viable in RMS for 5 days following aerobic incubation at 12 °C, as determined by sub-culturing. Interestingly, this is longer than what the same strain remained viable in faeces; which may be due to the removal of liquid from the manure/ slurry or possibly due to the other components of slurry such as urine and other bodily secretions which may aid survival. Thus further investigation into RMS as a DD infection reservoir is required.

In contrast, DD treponemes were not viable (as determine by sub-culture) following incubation in wheat straw and sand 5% (w/w) lime mix bedding, with DD treponemes not present at all immediately from the initial inoculation in the sand 5% (w/w) lime mix microcosm as judged through phase contrast microscopy. The poor level of presence and viability in straw of *T. phagedenis* DD treponeme phylogroup strain T320A is in contrast to many studies which have found straw to contain high loads of pathogens, particularly when soiled (Hogan et al., 1989; Ward et al., 2002; Yarnell et al., 2017). However, the lack of survival in straw does tie in with some risk factor studies which cite straw yards to be low risk for DD compared to other housing systems (Laven, 1999; Somers et al., 2003; Onyiro et al., 2008), however, this will also be in-conjunction with physical factors such as straw softness for foot health.

Adding hydrated lime to bedding is used as a means of helping to keep the bedding dry and control bacterial load as the lime increases the pH of the bedding and acts as a desiccant (Hogan et al., 1999; AHDB Dairy, 2014). In this study where sand had one of the best DD treponeme viability rates of the beddings investigated, the addition of approximately 5% (w/w) lime resulted in no survival of the DD treponemes almost immediately after inoculation. It is possible that the increase of pH caused by the addition of lime to sand may have result in the lack of DD treponeme survival in this bedding type as the pH may have reached pHs greater than pH 9 (Hogan and Smith, 1997) and survival of DD treponemes above this pH has not been investigated. However, it is much more likely that DD treponemes did not survive due to desiccation caused by the hydrated lime as

desiccation is effective against *Treponema pallidum* which causes syphilis in humans (Radolf et al., 2016). Thus lime may be a useful measure for the control of DD treponemes in other bedding types such as sawdust and RMS and further investigation is required along with investigation into concentration of hydrated lime required. However, lime must be used in moderation as it can cause skin irritation which may predispose the cow to disease (AHDB Dairy, 2014).

The survival of any bacteria in an environment is down to the interplay of many factors as opposed to just one factor including but not limited to temperature, pH, nutrient availability, oxygen concentration and moisture content (Godden et al., 2008). In the bedding and faecal microcosms, survival of DD treponemes in these environments was only investigated at 12 °C. A temperature of 12°C was chosen as it provided a good middle temperature between warm and cold temperatures experienced in the UK, which was readily achievable in the laboratory. However, different temperatures may have a positive or negative effect when in combination with other stresses in the environment microcosms on the ability of the DD treponemes to survive within faecal and bedding microcosms. Thus it would be interesting to investigate DD treponeme survival in environmental microcosms of different temperatures as these results may determine a time of year in which risk may be particularly high for bedding or faeces becoming a reservoir for DD treponemes.

Additionally, the difficult nature of culturing DD treponemes in the presence of other bacteria meant that faeces and bedding had to undergo sterilisation before use in the microcosms so that failure of DD treponemes to survive was reflective of the bedding and not on DD treponemes being outcompeted by other bacteria. Sterile bedding microcosms have also been successfully exploited in another bedding survival study (Godden et al., 2008). However, DD treponemes may have interactions with other bacteria that enable them to survive. Indeed, DD is a polymicrobial disease and DD treponeme phylogroups anecdotally prefer to grow together in culture and at least within the lesion (Evans et al., 2009c), suggesting a possible symbiotic relationship with other strains and species. Thus whilst the above data provides insight into individual DD treponeme phylogroup strain survival in different conditions, without the presence of other bacteria it is not fully representative of true DD treponeme survival in these conditions. Whilst, overcoming the difficulties of growing DD treponemes in culture with other bacteria may not currently be possible in enabling non-biased DD treponeme survival measurement, it may be possible to study mixed treponemal growth and survival with improved detection techniques specific

to each of the three cultivable DD treponeme phylogroups to accurately determine their levels within the microcosm.

Growth and motility scoring was carried out solely on DD treponemes which had typical spiral morphology, however, there is another morphology which was not accounted for. Like *Borrelia* and other spirochaetes (Brorson and Brorson, 1997; Mattman, 2001), DD treponemes are able to change their morphology to encysted forms (also known as a round or spherical bodies) (Chapter 1 Figure 1.3) (Walker et al., 1995; Evans et al., 2009b; Döpfer et al., 2012a); which may be linked to stresses in the environment (Murgia and Cinco, 2004), especially as they are first seen upon initial inoculation into culture (Chapter 1 Figure 1.3a) and when the spiral forms are reaching the stationary phase (Chapter 1 Figure 1.3b) (Walker et al., 1995; Döpfer et al., 2012a). These metabolically reduced encysted forms are reported to be able to revert back to spiral forms upon favourable conditions (Brorson and Brorson, 1997; Murgia and Cinco, 2004), and would therefore most likely have been accounted for when determining viability in microcosms using the sub-cultures of this study. The encysted form of *Borrelia* has been associated with the lack of efficacy of antibiotics in resolving Lyme disease in humans, and thus the encysted forms help to enable chronic disease (Murgia and Cinco, 2004). This draws parallel with DD treponeme persistence in the foot leading to chronic stage M4 DD and reoccurrence of DD lesions following antibiotic treatments (Berry et al., 2010; Döpfer et al., 2012a). It is also therefore likely, that under the stress of the survival study conditions in this study that DD treponemes became encysted. However, encysted forms of DD treponemes would have been difficult to measure in the environmental microcosms which contained a lot of particulate matter and would have confounded scoring. Further investigation is needed into the presence of encysted forms of DD treponemes in survival studies, their role in survival as well as suitable robust detection methodology for encysted DD treponemes, for example, a staining technique.

In summary, DD treponemes are able to remain viable in culture temperatures of 4, 12, 20 and 37 °C as well as in pHs of 5.5 to 9. Exact ranges for viability and conditions optimum for growth varied between DD treponeme phylogroup and strains within phylogroups. In faecal microcosms incubated aerobically at 12 °C *T. phagedenis* DD treponeme phylogroup strain T320A and *T. pedis* DD treponeme phylogroup strain T3552B were viable for a median of 1 day. The range in number of days viable between experiments meant that for T3522B there was the possibility that it may survive for up to 6 days in this environment. Of the five types

of bedding assessed for *T. phagedenis* phylogroup strain T320A survival and viability, straw bedding or sand supplemented with 5% (w/w) lime appeared to prevent survival whereas RMS enabled viability for 5 days maximum, sand for 6 days and sawdust enabled T320A to remain viable for the full duration of the study. These results indicate that bedding could be a potential infection reservoir for DD. These findings not only help to predict where DD treponeme reservoirs may be found, they point to methods for controlling DD treponemes in the environment. However, implementation of any control strategies should take into account the effect of the strategy upon other pathogenic microorganisms, for example, changing to straw bedding. Additional investigation into DD treponeme survival under varying conditions may be required to definitively define duration of survival, for example investigations into the effects of other factors such as temperature, moisture content and so forth on environmental microcosms. Furthermore of interest would be additional bedding types to those investigated here and faeces from cattle on various diets. This work provides a foundation for future DD treponeme survival studies.

Chapter 7: Discussion

7.1 The importance of identifying DD infection reservoirs

The overarching aim of this project was to identify and better understand the possible infection reservoirs of DD with regards to treponemes. Treponemes have consistently been identified within DD lesions and are thus considered to be integral in DD aetiology. By identifying infection reservoirs, the routes of transmission can begin to be pieced together and through this knowledge prevention strategies can be developed which are tailored to the infection reservoirs of DD and DD-associated treponemes specifically. Current treatment methods of topical sprays and footbaths are not specifically targeted to DD treponemes and only focus on the DD lesions, as previously they were the only known source of infection (Laven and Logue, 2006; Evans et al., 2009a, 2012a; Yano et al., 2010a; Angell et al., 2015a). Therefore reoccurrence of DD is inevitable and elimination of DD on farms has not been possible, resulting in DD continuing to be a high welfare and economic burden on the dairy industry. Only in the past 20 years, with the advent of better detection techniques, has the role of DD treponemes in DD aetiology been explored and there is still much to discover; including how they contribute to DD pathogenesis and how transmission occurs. Additionally, the recent emergence of new forms of DD in other species (Dhawi et al., 2005; Moore et al., 2005; Sullivan et al., 2013, 2014b; Clegg et al., 2015) and identification of DD treponemes in other lesion types in dairy cattle and pigs (Pringle et al., 2009; Evans et al., 2010, 2011a; Svartström et al., 2013; Clegg et al., 2016a; c; d; e) has further driven the need for new prevention strategies and identification of infection reservoirs.

Until recently the only information about how DD may be spread, other than from direct contact with a DD lesion, was through risk factor studies which predominantly identified hygiene as a high risk factor for DD. With the subsequent determination of a polytreponemal aetiology, studies have begun to dissect the dairy farm environment as well as dairy cattle tissues for possible infection reservoirs and with identification of DD treponemes in sites other than lesions, transmission routes are beginning unfold (Chapter 4 Figure 4.7).

7.2 Identification of infection reservoirs and implications for disease prevention

7.2.1 Infection reservoirs in the GI tract

An initial study investigating DD infection reservoirs found for the first time that DD treponemes were associated with tissues in the oral cavity, rumen and rectum of dairy cattle (Evans et al., 2012b). More recently two other studies found DD treponemes to be associated with rumen fluid and another confirmed the presence of DD treponemes in sheep and beef oral and RAJ tissue (Nascimento et al., 2015a; Sullivan et al., 2015a; Zinicola et al., 2015b). The studies in Chapters 4 and 5 expanded upon the work by Evans et al., (2012b) by surveying a larger number of dairy cattle. In Chapter 4 the prevalence of DD treponemes in the gingiva and RAJ were slightly lower than in the initial study and more in line with what was found in beef cattle and sheep (Sullivan et al., 2015a), although in this study no seasonal associations were found. Additionally, cultures of the gingiva and RAJ containing spirochaete-like morphology were deemed positive by DD treponeme phylogroup specific 16S rRNA gene PCR assays for one or more of the three DD treponeme phylogroups (Chapter 5); suggesting that DD treponemes are viable in these tissues as previously described by the isolation of a *T. phagedenis* phylogroup treponeme from a sheep RAJ (Sullivan et al., 2015a). Histopathology of a small number of gingiva and RAJ tissues positive for DD treponemes showed no disease pathology, suggesting that carriage in these tissues does not cause disease (Chapter 5), although larger numbers of DD treponeme positive GI tract samples would be required to confirm this observation which may be achieved by conducting a much larger GI tract survey than carried out in this study. Moreover, *T. medium* and *T. phagedenis* phylogroup STs obtained by MLST in the GI tract were predominantly the same as those found in DD lesions from dairy cattle and other species (Chapter 5). However, as for the histopathology, a larger number of GI tract samples would need to be surveyed to obtain a better representation of STs present in the GI tract with comparison to DD lesions and other tissues.

Whilst beginning to answer some questions about the GI tract as a potential infection reservoir of DD, such as whether the same DD treponemes within a phylogroup involved in DD are colonising the GI tract, are these treponemes viable and is colonisation of the GI tract causing damage to the tissue? There are still many questions unanswered. A limitation to the way in which cows were obtained for this study meant that herd

information and management practices of the cows were unavailable, therefore certain associations were difficult to deduce. For example, there appeared to be no association between GI carriage of DD treponemes and the housing season, although previously a positive association had been described (Evans et al., 2012b). Therefore it is unknown whether the cattle sampled had access to pasture or were housed all year. If housed all year, presence in the GI tract may not necessarily be associated with only the winter months when cows would be typically housed and there has been a reported moderate increase in the housing of animals across the last ten years (March et al., 2014). Furthermore, where GI tract samples colonised by DD treponemes came from cattle not affected by DD, it would have been interesting to know whether these cattle came from herds which had no DD prevalence or whether it was a herd in which DD was endemic.

Future infection reservoir surveys would be required which sampled from herds where the herd history could be obtained. Useful information to collect would include herd DD status, housing systems, farm management practices, prevalence of other diseases (especially those in which DD treponemes have been implicated e.g. non-healing foot disorders or hock lesions) and diets of the cattle sampled. Such data could then be mined for associations with not only GI tract presence but other infection reservoirs, and could pinpoint high risk practices resulting in infection reservoir occurrence. However, as described earlier a different sampling method to using carcasses from a fallen stock disposal service would be required for this information to be obtained. Indeed a route to overcome this sampling limitation would be to develop a method to monitor DD treponeme presence in live cattle, perhaps through the use of swabs together with a longitudinal survey of a natural outbreak. However, it would be important to do further studies that aim to determine the localisation of DD treponemes in the GI tissues as swabs may not pick up DD treponemes which predominantly localise deep within the tissues. This study using IHC failed to determine DD treponeme localisation in GI tissues as no labelling occurred in the small number of DD treponeme PCR positive tissues investigated.

Sampling from live animals would enable colonisation of the GI tract of individual animals to be monitored to determine whether colonisation is permanent or transient as for *E. coli* O157 in cattle (Naylor et al., 2003; Keen et al., 2010), as well as determine possible microbiome changes associated with DD treponeme presence in the GI tract compared to non-colonised cattle using metagenomics. In addition, it may be possible to deduce a timeline for colonisation in different tissue types by monitoring heifers either in DD

endemic herds or possibly by introduction of DD to a naive herd (Chapter 4 Figure 4.7). This may clarify whether colonisation of the feet occurs first and then GI tract colonisation occurs or vice versa as well as in what order different GI tract tissues become colonised. Furthermore, the identification of cows containing DD treponemes in one or more tissue types would enable investigations into how that cow may enable transmission of DD treponemes by monitoring it and what it comes into contact with. Indeed, there has been one attempt to monitor the longitudinal progress of a dairy cattle DD outbreak in cattle in relation to the GI tract infection reservoir although unfortunately the disease failed to be transmitted (Capion et al., 2013).

A further limitation of this study was sampling of only one site of the gingiva or RAJ from each cow. This presumes that DD treponeme colonisation would be ubiquitous throughout that anatomical location i.e. encompassing the entire RAJ. Whilst Evans et al., (2012b) demonstrated that DD treponeme presence in the gingiva between the lower molars did not occur, even if DD treponemes were present between the upper molars of the same cow; it may be possible that only one side of the oral cavity is positive. Thus further investigation may be required to determine if heterogeneous DD treponeme colonisation occurs by sampling multiple sites for each tissue type to prevent a possible underestimation of DD treponeme prevalence in these tissues.

Rumen fluid had not yet been identified as a potential infection reservoir for DD when this study commenced and thus was not investigated, however, it would be interesting to gain a better understanding of the prevalence of cows with DD treponemes in rumen fluid and other tissues of the rumen by conducting an exploratory survey. Rumen tissues of a single cow in which DD treponemes were detected in the Evans et al., (2012b) study were also positive for the same *T. phagedenis* DD treponeme phylogroup in the gingiva and these samples were analysed by MLST in this study which revealed that the same *T. phagedenis* phylogroup STs were found in the rumen dorsal sac and gingiva, although a different ST was found in the rumen reticular pillar, although this may be due to a bias for more dominant STs from applying MLST direct to tissues. Thus further study into whether rumen tissue and rumen fluid colonisation is linked to each other and to colonisation in other sites of the GI tract would be beneficial, particularly as it has been postulated that presence in rumen fluid could account for presence in the gingiva or vice versa due to leakage of rumen fluid into the oral cavity (Chapter 4 Figure 4.7).

How DD treponeme colonisation of the GI tract contributes to DD maintenance on farms is currently unknown, although it has been demonstrated that DD treponemes can be viable in these tissues (Sullivan et al., 2015a). It is speculated that carriage in the RAJ may result in shedding in faeces (Chapter 4 Figure 4.7). However, this link has not been proven. Additionally it has been postulated that rumen fluid may be leaked from the mouth resulting in contamination of the environment if the rumen fluid contained DD treponemes, however, it is unknown whether DD treponemes in rumen fluid are viable as currently only molecular studies have identified their presence (Nascimento et al., 2015a; Zinicola et al., 2015b). Again, it would be useful to collect data from live animals during a longitudinal study to understand the contribution of these treponeme infection reservoirs to transmission, for example, the identification of DD treponeme colonisation in the RAJ of particular cows would enable targeted investigation into the faeces of these cows to determine if the DD treponemes are being shed in faeces. Furthermore actual bacterial isolation from these niches would further support the potential for transmission from them.

Currently, without the ability to easily detect DD treponemes in the GI tissues of live cattle it would be difficult to control for cattle entering a heard which carried DD treponemes in their GI tract. However, if the GI tract is indeed important for DD maintenance on farm it is important that DD treponeme colonisation is addressed. Ideally, one method would be to quarantine cattle with DD treponemes in the GI tract and treat for DD treponemes, possibly through systemic antibiotics, depending upon whether further investigations into their efficacy are undertaken (Laven and Logue, 2006; Evans et al., 2016). However, to minimise the use of antibiotics cattle could be treated with probiotics as with other diseases (Robbins et al., 2013), but investigations into the efficacy of this treatment would also be necessary.

7.2.2 DD treponemes in healthy foot tissue

Previously, a very small number of non-DD lesional pedal tissue were detected as positive for the presence of DD treponemes by molecular methods (Evans et al., 2009c, 2012b). This prompted a larger survey of this tissue type in this project and surprisingly DD treponemes were detected in 18.9% of healthy foot tissues analysed by a combination of molecular and culture methods. Further investigation lead to the majority of these tissues showing histopathological changes consistent with an infection and MLST showed that many of these tissues contained STs associated with DD lesions (Clegg et al., 2016b). Whilst it

cannot be proven that changes in the tissue were as of a result of the DD treponeme presence it could be postulated that they are sub-clinical DD lesions and may have progressed into visible M1 lesions had they been detected in live cattle (Rasmussen et al., 2012). DD treponemes were shown by IHC to be on or near the surface of some of these healthy foot tissues by IHC and thus cattle which do not yet show signs of DD may still be able to spread DD treponemes (Chapter 4 Figure 4.7).

However, two healthy foot tissues in which DD treponemes were detected by PCR showed no histopathological changes associated with an infection. In addition a treponeme belonging to the *T. phagedenis* DD phylogroup was isolated from one of the tissues with no changes proving, for that tissue at least, the DD treponemes were viable. Whether colonisation was truly resulted in no tissue changes requires further investigation as it is possible these samples were taken very early in infection and changes had not yet occurred.

Future studies sampling from live cows, as described in Section 7.2.1, would enable follow up of any skin samples with no DD lesions visible which were positive for DD treponemes (with or without histopathological changes suggesting infection) to observe whether they progressed into M1 DD lesions or remained healthy despite DD treponeme presence. In addition, studies to dissect the importance of skin containing DD treponemes which exhibit no visible DD lesions are needed. Studies could investigate how such skin may act as an infection reservoir, including additional surveys of fomites before and after they have come into contact with these DD lesion-free DD treponeme positive feet to determine whether DD treponemes are transferred.

7.2.3 Faeces as an infection reservoir

The presence of DD treponemes in the RAJ (Evans et al., 2012b; Sullivan et al., 2015a) and hygiene as a major risk factor for DD (Rodríguez-Lainz et al., 1996; Hultgren and Bergsten, 2001), lead to the hypothesis that DD treponemes were shed in the faeces. Together with the fact that slurry causes maceration of the skin required for the experimental induction of DD, the possible presence of DD treponemes in faeces would provide further stimulation for better hygiene practices. Thus faeces from dairy cattle were investigated again in this project for the presence of DD treponemes.

Following suit from previous studies (Nordhoff, 2006; Evans et al., 2012b; Sullivan et al., 2015a), despite using an improved DD treponeme detection method for faeces (Chapter 3),

detection of DD treponemes failed using PCR directly on DNA extracted from dairy cow faeces (Chapter 4). However, metagenomic studies employing more sensitive sequencing approaches have been able to detect a low abundance of DD treponeme DNA in faeces and slurry (Klitgaard et al., 2014, 2017; Zinicola et al., 2015b). The very low abundance of DD treponemes in slurry and faeces has lead to debate as to the importance of faeces/slurry as a vehicle for transmission (Klitgaard et al., 2017), especially as it was unknown if the treponemes were viable and thus transmissible.

For the first time in this study (Chapter 4), a DD treponeme was isolated from a faecal sample and 16S rRNA gene sequencing determined it to belong to the *T. phagedenis* DD treponeme phylogroup, demonstrating viability of the treponemes in faecal material. The same faecal sample was negative by direct PCR on the faecal sample. It is likely that the sensitivity of the PCR may have been too low for detection before growth of the DD treponeme in culture or that faecal inhibition of the PCR was occurring which was negated by PCR of culture. Additionally, another faecal sample collected from a different cow demonstrated spirochaete-like morphology upon cultivation, although isolation failed. DD treponeme specific PCR assays carried out on the culture of this faecal sample detected *T. phagedenis* DD treponeme phylogroup DNA within that faecal culture (Chapter 5). This sample was submitted for MLST, however, the *T. phagedenis* phylogroup ST allocated had not been observed previously in DD lesions from any species (Clegg et al., 2016b). This raises the question as to whether the DD treponemes found in faecal material are the same STs involved in DD lesions. A larger MLST study of faecal samples would be required to determine if this was true, although due to the prior difficulties of isolating DD treponemes from faecal samples MLST direct from mixed culture may be necessary.

Although DD treponemes have been detected in faeces, now by both molecular and cultivation methods, their role as infection reservoir is still perplexing as survival times in this niche was unknown and could be considered key to enable transmission. To try to shed light on this question, survival of each of the three DD treponeme phylogroups was evaluated in bovine faecal microcosms. This study found that the *T. phagedenis* and *T. pedis* DD treponeme phylogroup were viable in sterile faeces for a median of one day (range of 1-3 and 0-6 days respectively). However, growth in faeces from what was originally inoculated was not noticeable within the microcosms suggesting that proliferation of the treponemes would not occur in faeces and thus the amount of treponemes in faeces would be dependent on the amount shed in faeces and also through

direct lesion contact with slurry. Further investigation into survival of DD treponemes in relation to faecal composition with diet, survival in faeces with other bacteria present or in mixed phylogroups as well as in different temperatures that may occur on farm would be needed to fully delineate the survival of DD treponemes in faeces. Additionally investigation into the survival of DD treponemes in slurry is warranted, especially as slurry contains components in addition to faeces such as urine which may aid or inhibit DD treponeme survival compared to survival in faeces alone.

Whilst survival may appear quite short in faeces, it is highly likely other cattle will come into contact with this DD treponeme infected faeces during this time, particularly when walking through slurry in walkways and chutes. Additionally, if machinery or farm personnel boots and equipment are contaminated with faeces/slurry and they move between herds or operations within a day, they are at risk of transferring DD treponemes to a different herd or operation. This gives credence to the biosecurity risk factors for DD outlined by Oliveira et al., (2017), for example the increased risk of DD when boots are not provided for visitors and when slurry scraping machinery is used for other purposes.

Whilst abundance of DD treponemes appears to be low in faeces and slurry, further investigation into abundance levels in different herds may be required. DD treponemes have thus far only been found in faeces/slurry on farms endemic for DD (Klitgaard et al., 2014, 2017) and thus far only a small number of cattle have been identified as carrying DD treponemes in the RAJ, although it is unknown whether they came from DD endemic farms. If farms were identified that had a higher prevalence of DD treponemes in the RAJ, investigation of slurry for DD treponeme abundance may find higher levels of abundance in these farms, which may also help to link RAJ presence with faecal shedding. Although, further information is needed to determine the role of slurry and faeces as a primary infection reservoir in DD spread, awareness of the possible risks for DD faeces/slurry poses and maximising strategies to increase hygiene can only be beneficial.

7.2.4 Increasing evidence of fomites as DD infection reservoirs

Sullivan et al., (2014a) recently detected DD treponemes on the blades of hoof trimming knives providing microbiological evidence for the risk of increased DD relating to the frequency of foot trimming and the hygiene practices involved (Wells et al., 1999; Holzhauer et al., 2006). This data along with other studies investigating oral treponeme adherence to metal orthodontic brackets suggests that DD treponemes are able to adhere

to metal surfaces, but what the Sullivan et al., (2014a) study also suggests is that DD treponemes can easily be transferred from the foot to another surface (Chapter 4 Figure 4.7). This lead to the question of what other surfaces does the foot come into contact with which may enable DD infection?

Investigations into this question in this project (Chapter 4) lead to the detection of DD treponemes on gloves used to handle DD-affected dairy cattle feet during foot trimming, which has also been recently identified with gloves used to handle CODD-affected sheep (Angell et al., 2017). Additionally DD treponemes were detected on a small number of foot trimming tools (clippers and hoof grinder), however sample size of each tool was small and thus future surveys using a larger sample population would be needed to understand the true prevalence of DD treponemes on these fomites and enable more powerful statistical associations. For the first time DD treponemes were shown to be prevalent in footprints left behind on the floor where dairy cattle had been standing for a period of time (i.e. the milking parlour and crush floor). This data is therefore highly suggestive that DD treponemes are being transferred to fomites from the foot and thus through indirect contact transmission of DD may be occurring (Chapter 4 Figure 4.7). It would therefore also be important to discover which other fomites may harbour DD treponemes following contact with either cattle feet or fomites such as gloves and trimming tools.

What was not investigated was the contact time which was needed for DD treponemes to be imparted onto a surface, such as the floor, from the foot. Whilst this may be arbitrary in terms of cattle standing in the parlour to be milked or trimmed in a crush, as the time in which standing occurs cannot be changed, a short contact time would suggest that DD treponeme transference may also occur when cattle are walking and this needs to be investigated as more surfaces may then harbour DD treponemes such as walkways. This may make control of footprints as a possible route of transmission particularly difficult and focus would have to be on making sure the foot is clear of DD treponemes in the first place which may be difficult if there are no clinical signs of DD and with current treatments not being optimal for DD treponemes.

The recent data also suggests that it is not just metal surfaces that are a risk for possible DD adherence. Gloves, hoof trimming handles (wrapped in a cohesive bandage, see Chapter 4 Figure 4.3b), and footprints on rubber and concrete flooring all had DD treponemes detected on them. Interestingly, there was a higher prevalence of DD treponemes detected on rubber and concrete floors as opposed to metal. Thus metal should not be the only

focus for DD treponemes on fomites. Concrete is one of the most popular floor surfaces used on farms and various types are often cited as risk factors for DD, which is thought to be due to slurry retention and mechanical effects on the hoof (Wells et al., 1999; Hultgren and Bergsten, 2001; Somers et al., 2005). However, many farms are moving away from metal and concrete surfaces in favour of rubber flooring for cattle comfort and protection from mechanical claw damage (Vokey et al., 2001; Telezhenko et al., 2007; Ouweltjes et al., 2009; Fjeldaas et al., 2011; Eicher et al., 2013). Therefore the findings in this project should raise awareness of the microbiological implications of floor material choice and that management of floor hygiene is imperative.

Hygiene is particularly important, as although not investigated in this study, viable DD treponemes have been identified on both gloves and a foot trimming blade (Sullivan et al., 2014a; Angell et al., 2017). This demonstrates that the DD treponemes on these fomites are viable and could therefore infect another foot. Ideally, future work is needed to determine if DD treponemes are viable on all fomites investigated (e.g. footprints on floor surfaces) to assess whether they are true infection reservoirs and thus how important they may be for transmission. This is dependent on the ability of DD treponemes to be cultured from these fomites as if future studies are unable to isolate from these fomites it may be more reflective of the difficulties in culturing DD treponemes than their actual viability on the fomites in question.

Survival of DD treponemes over time on these fomites was not investigated in this study or in the study by Sullivan et al., (2014) but was investigated for gloves used to handle CODD-affected sheep and thus could also be applied to DD treponemes on gloves from handling clinical cases of DD in dairy cattle (Angell et al., 2017). The study found that DD treponemes were viable on gloves for one to three days depending upon DD treponeme phylogroup (Angell et al., 2017). Thus, not only would DD treponemes remain viable between cattle if gloves were not changed but if contaminated gloves were not disposed of correctly they could continue to contaminate other objects for up to three days which may then be used on other operations within that time. Survival of DD treponemes on foot trimming tools and floor surfaces must also be assessed to determine the risk imposed by these fomites as infection reservoirs on and between farms. Furthermore, determining the survival of DD treponemes on different surface materials (e.g. metal or rubber) can inform upon which material poses the greatest risk for becoming an infection reservoir and this knowledge may be utilised in control strategies. Work is currently underway at the University of

Liverpool to assess survival on foot trimming knife blades. If survival is for a similar duration as with gloves it would further substantiate microbial evidence for risk factors imposed by foot trimming and professionals who work on multiple operations (Wells et al., 1999; Holzhauer et al., 2006; Oliveira et al., 2017).

For gloves, changing and adequately disposing of gloves between cattle, as well as minimising what is touched by the gloves whilst wearing them should help to adequately control this infection reservoir. Where equipment is not disposable, e.g. foot trimming tools and floor surfaces, methods to remove DD treponeme contamination should be considered. Ideally, equipment and floors should be disinfected with a disinfectant that is effective on DD treponemes, will not affect the integrity of the materials it is applied to, be environmentally friendly and low health risk to animals and humans in the vicinity. Furthermore, for successful uptake, the disinfectant should be cost effective, low labour and fast acting to minimise time loss.

A small number of studies have begun to investigate possible useful disinfectants directed at DD treponemes. An iodine based disinfectant was shown to have some effect but did not completely prevent DD treponeme detection on foot trimming blades by molecular methods (Sullivan et al., 2014a). Whether the treponemes were still viable is unknown, as only a small number of the total swabs taken were investigated by culture following disinfection of the blade (Sullivan et al., 2014a). Angell et al., (2017) investigating various different disinfection methods found that plain water was completely ineffectual. However, a 1 in 90 dilution of FAM or 70 per cent ethanol were the most effective methods for decontaminating the gloves when assessed for DD treponeme detection by both molecular and culture methods (Angell et al., 2017). Furthermore, a study has investigated the minimum inhibitory concentrations of disinfectants used in footbaths for a *T. phagedenis* DD treponeme phylogroup isolate (Hartshorn et al., 2013), and the results obtained could be further explored and applied to disinfection of floors and equipment. In addition faeces can affect the efficacy of disinfectants in footbaths and this must be borne in mind when disinfectants are analysed (Hartshorn et al., 2013), especially for the floor of crushes and parlours which can become heavily soiled with faeces. Moreover, certain disinfection practices may not be appropriate for all types of foot trimming equipment, for example, those which have lubricants or are powered electrically (Sullivan et al., 2014a). Thus further work is needed, incorporating the knowledge already obtained from previous studies, to

address which disinfectant is the most effective against DD treponemes for each type of fomite and the farm conditions they may be subject to such as heavy soiling.

Although bedding frequently comes into contact with the foot and is known for harbouring other pathogens, it has not previously been considered as a possible DD infection reservoir and does not feature in many risk factor studies, despite more farms investing in RMS bedding. Given that DD treponemes have been detected in faeces/ slurry and can survive a median of one day in faeces, RMS as a bedding type is of particular concern as a possible infection reservoir (Chapter 6). Whilst not surveyed for DD treponemes in this project, it was found that DD treponeme *T. phagedenis* strain T320A had variable viabilities in different sterile bedding types (Chapter 6). DD treponemes appeared to be viable for longest in sand (seven days), followed by sawdust (six days) and then RMS (five days). This data suggests that these bedding types could be possible infection reservoirs for DD treponemes. This study did not investigate the bedding with other bacteria present, in different temperatures or soiled bedding, which may positively or negatively affect DD treponeme viability and thus warrants future investigation. Additionally it did not delve into why certain bedding types may be better or worse such as inherent moisture content or pH which could also be measured in future survival studies. Importantly, this data indicates that a survey for DD treponemes in bedding is warranted due to their ability to survive in certain bedding types and the fact they have been found on surfaces that come into contact with the feet in this study and elsewhere (Sullivan et al., 2014a; Angell et al., 2017). Furthermore, investigation of bedding type used on farms as a risk factor for DD may further consolidate the importance of bedding as a possible infection reservoir.

Interestingly, DD treponemes did not appear viable in straw or sand mixed with 5% (w/w) lime and the results for straw support risk factor studies that find DD prevalence is less in straw yards (Laven, 1999; Somers et al., 2003; Onyiro et al., 2008). Thus should bedding type be confirmed as an infection reservoir, these beddings may help to control for DD treponemes and thus reduce the risk of DD transmission from bedding. The ability of DD treponemes to survive in sand but not sand with the addition of hydrated lime, which acts as a desiccant in addition to producing an alkaline environment, highlights desiccants and/ or alkaline environments as a possible means of controlling DD treponemes. Further survival studies could be conducted to determine whether other desiccants such as ash inhibit DD treponeme survival, what concentration of desiccant is required to prevent DD treponeme survival and whether it is as effective when added to bedding types other than

sand such as sawdust. Additionally further work is needed to investigate DD treponeme survival with other materials which may produce alkaline environments for use with bedding.

The presence of DD treponemes on fomites that come into contact with the foot is a worrying trend and further investigation is required to a) determine the viability of DD treponemes on these fomites. b) assess the role of these fomites as an infection reservoir in terms of ability to allow transmission and relative importance. c) determine what other fomites might be contaminated with DD treponemes and thus be a possible infection reservoir and d) how to effectively eliminate these fomites as infection reservoirs.

7.2.5 Other considerations for DD treponeme infection reservoir identification

The identification of DD treponemes in both pedal tissue and the GI tract raises the question as to where else DD treponemes may be found in the host that has not already been explored and how DD treponemes may come to colonise these tissues, especially within the GI tract. Spirochaetaemia (the presence of spirochaetes in the blood) occurs in other spirochaetal infections including *Treponema pallidum* or *Borrelia burgdorferi* infections in humans (Lee et al., 2010; Radolf et al., 2016) . Indeed, two phylogroups of DD treponemes (*T. medium* and *T. pedis*) display β-haemolysis on blood agar (Evans et al., 2008) and *T. phagedenis* has been shown to have an ortholog of pallilysin which can degrade proteins involved in blood coagulation and basement membranes, although it appears inactive in *T. phagedenis* (Houston et al., 2015). It could therefore be postulated that DD treponemes are disseminated in the blood stream which may lead to their eventual colonisation of the GI tract from DD lesions or vice versa under specific conditions (e.g. metabolic stress). Exploratory work could be undertaken to determine whether DD treponemes can be detected in the blood during different stages of DD lesion development which may provide further insight to infection reservoir dynamics and the maintenance of DD on farm.

The polytreponemal aetiology of DD has meant that investigations into infection reservoirs of DD have focused solely on treponemes, however, DD is also a polymicrobial disease and the exact roles of treponemes and how they interact with other bacterial genera for DD lesion development and progression has yet to be elucidated. Future investigations into infection reservoirs may also look at the presence of DD treponemes along with the presence of other taxa commonly identified in the DD lesion microbiomes such as

Mycoplasma spp. or *Porphyromonas spp*. (Chapter 1 Table 1.1) (Berry et al., 2010; Santos et al., 2012; Krull et al., 2014; Zinicola et al., 2015b; Nielsen et al., 2016). Indeed, some studies have investigated the presence of other bacteria in infection reservoirs as well as DD treponemes with one study looking at the general microbiome of slurry as well as targeting DD treponemes (Klitgaard et al., 2017). Another study focussing on gloves as a treponeme infection reservoir in relation to contagious ovine digital dermatitis (CODD) also investigated the presence of *Dichelobacter nodus and Fusobacterium necrophorum* due to the close association of ovine foot rot with CODD (Angell et al., 2017). Identification of whether particular bacterial species found in DD lesions also associate with DD treponemes in infection reservoirs may aid in better understanding of the how DD treponemes interact with other bacterial species in DD lesion development and progression.

Improving detection techniques for DD treponemes would underpin future studies into DD treponeme infection reservoirs. Molecular techniques have been one of the most important tools for DD treponeme detection in infection reservoirs due to the difficulties in culturing DD treponemes as a result of their fastidious nature. In this thesis PCR methodology specific for the three cultivable DD treponeme phylogroups (*T. medium, T. phagedenis* and *T. pedis*) was utilised from previous studies investigating DD treponemes (Evans et al., 2009c, 2012b) for several reasons. Firstly this methodology had previously been successful in determining the associations of these phylogroups in DD lesions (Evans et al., 2009c) and had also successfully detected DD treponemes in other cattle tissues (Evans et al., 2012b). Secondly, conventional PCR is relatively cheap and thus a large number of samples can be processed without too much expense. Thirdly the infection reservoir survey conducted here was primarily exploratory and thus only presence/ absence data was required. However, for further investigation of DD treponeme infection reservoirs detection methods which are more sensitive and would enable more information such as quantification to be obtained would be most useful.

Real time PCR assays specific for each of the DD treponeme phylogroups would still enable large numbers of samples to be easily processed relatively cheaply but would also enable the collection of qualitative and/or quantitative information about the presence and abundance of these DD treponeme phylogroups in samples investigated. In addition, real time PCRs are more sensitive and specific than conventional PCR assays due to the measurement of amplification during the exponential phase using fluorescence. Such enhanced sensitivity may allow DD treponeme detection where concentrations are very

small such as faeces. Furthermore using real time PCR it could be possible to determine how important an infection reservoir may be by the quantity of DD treponemes detected within it and also determine which is the most prevalent phylogroup if multiple phylogroups are detected. Real time PCR assays specific for the *T. medium, T. phagedenis* and *T. pedis* DD treponeme phylogroups have recently been developed which were tested on DD treponemes from culture (Anklam et al., 2017). This real time methodology could be applied to a range of infection reservoir material in the future to further delineate the infectious cycles of this important infectious lameness of cattle.

Metagenomic sequencing techniques are more sensitive than PCR assays, can be used to determine the treponemal microbiome and/ or general microbiome of a sample and have enabled molecular detection of DD treponemes direct from faeces where PCR methodology has previously failed (Evans et al., 2012b; Klitgaard et al., 2014, 2017; Sullivan et al., 2015a; Zinicola et al., 2015b). However, metagenomic sequencing is more expensive than PCR methodologies and is more time consuming in terms of data analysis and thus metagenomic sequencing of a large number of samples for DD treponeme prevalence on an exploratory basis is not feasible. However, metagenomic sequencing could be used in future studies to investigate the treponemal and general microbiomes of known DD infection reservoirs and thus determine which taxa are most prevalent in these samples compared to DD lesions, as has been carried out for faecal and rumen samples (Klitgaard et al., 2014, 2017; Zinicola et al., 2015b).

7.3 Insights into DD treponemes

The fastidious anaerobic nature of the host associated DD treponemes may have lead to an underestimation of how versatile these motile bacteria can be and thus hampered investigations into their spread. The detection of DD treponemes in the environment (Klitgaard et al., 2014, 2017; Sullivan et al., 2014a; Zinicola et al., 2015b; Angell et al., 2017), especially on a range of fomites, where oxygen concentration may be high, nutrients low and temperatures below body temperature is highly surprising. However, with the fast spread of DD within herds, between herds and into new species these findings of versatility provide a plausible explanation as to why this spread has occurred.

Along with the detection of DD treponemes in the environment in this study, the survival of DD treponemes under various conditions gave further insights into their versatility with DD

treponemes remaining viable (as determined by secondary culture) in temperatures down to 4°C and in pHs as low as 5.5 and as high as pH 9.

Interestingly, these studies also demonstrated there is diversity in growth requirements between and within phylogroups. Whilst all DD treponemes investigated demonstrated overarching similarities in their ability to survive at different temperatures there was some small differences in optimum pH, pH range for good growth and duration of survival at different temperatures. For example, The *T. phagedenis* DD treponeme phylogroup strain T320A remained viable at 45 °C for two days as determined by secondary cultures, whereas the other two phylogroups showed no viability past initial inoculation into the microcosm. Additionally, the pH which was optimum for growth for *T. phagedenis* phylogroup strains T320A and T354B were different.

These differences may be important for DD treponeme detection via culture as DD treponemes are all cultured in the same conditions except for the *T. medium* DD treponeme phylogroup requiring a different serum. This may have lead to bias in detection, especially for *T. phagedenis* DD treponemes to which the current culturing conditions are most suited. In this study, the *T. phagedenis* DD treponeme phylogroup was the only DD treponeme phylogroup that was isolated from tissues and was the first DD treponeme phylogroup to be identified in faeces by culture. Similarly when DD treponemes were isolated for the first time from a foot knife blade (Sullivan et al., 2014a) and the RAJ (Sullivan et al., 2015a) of a sheep they belonged to the *T. phagedenis* DD treponeme phylogroup. Other studies also seem to have preferentially used *T. phagedenis* DD treponeme isolates from DD lesions in their studies suggesting they are the most readily isolated (Hartshorn et al., 2013; Wilson-Welder et al., 2013). In the UK and USA, only the *T. medium, T. phagedenis* and *T. pedis* DD treponeme phylogroups have been regularly detected by culture but metagenomic studies have shown other treponeme phylotypes of *Treponema paraluiscuniculi, Treponema maltophilum, Treponema putidum, Treponema refringens* and *T. denticola* are associated with DD in Denmark, Brazil and the USA (Klitgaard et al., 2014; Krull et al., 2014; Zinicola et al., 2015b). With the diversity shown in optimum conditions for growth between the three cultivable phylogroups it is likely that culturing conditions may not be optimum for these species either, preventing successful cultivation.

The knowledge gained from these survival studies may be used to try and optimise for growth of particular DD treponeme phylogroups which may in turn improve isolation of

these treponemes from potential infection reservoirs. Further work would be required to determine the optimum culture temperature of each of the cultivable DD treponeme phylogroups (*T. medium, T. phagedenis* and *T.* pedis), as in this study only a small number of temperatures were investigated across a wide temperature range. However, the data from this study does narrow down the temperature range that should be investigated as visible growth only occurred in the microcosms incubated at 37 °C, although DD treponemes remained viable at the other temperatures investigated. Studies investigating the optimum temperature range should be mindful of DD lesion temperatures determined to be between 27 and 30 °C (Stokes et al., 2012b), the core body temperature of cattle which has a narrow range around 39 °C (Suthar et al., 2011) and the temperature ranges in which growth has been observed for *T. phagedenis, T. vincentii* and *T. denticola* (Brenner et al., 1984). Following the determination of optimum temperatures for each of the phylogroups, the growth curves of each phylogroup and different strains within each phylogroup could be investigated when cultured in the determined optimum temperatures and pHs to gain a better understanding of growth in these conditions for culture maintenance and isolation methods.

Overall the *T. medium* DD treponeme phylogroup appeared to be the most sensitive to changes in conditions. When investigating DD treponeme survival in faeces incubated aerobically at 12°C, the *T. medium* DD treponeme control that was not in faeces but also incubated under the same conditions was also not viable past initial inoculation (although the *T. medium* DD treponeme phylogroup stock used for inoculations remained viable when incubated anaerobically at 36°C). In comparison the other two phylogroup controls remained viable when incubated in these conditions for a median of six days (as determined by secondary culture). DD treponemes have been shown in this project to be viable at 12°C for at least 21 days for the *T. phagedenis* and *T. pedis* phylogroups and 15 days for the *T. medium* DD treponeme phylogroup. Therefore the difference in survival between the phylogroup controls is likely due to the *T. medium* DD treponeme phylogroup having a different level of aerotolerance to the *T. phagedenis* and *T. pedis* DD treponeme phylogroups. Aerotolerance of the DD treponemes was not investigated in this study but in light of these survival results and detection in the environment further investigation is required into the survival of DD treponemes in different oxygen concentrations. However, this data does suggest the *T. medium* DD treponeme phylogroup may be less of a threat for DD infection in the environment unless it is protecting itself, for example through biofilm

formation. DD treponemes and their possible involvement in biofilms is currently under investigation at the University of Liverpool.

The DD treponemes did not appear to survive at temperatures above 45°C, although the *T. phagedenis* DD treponeme phylogroup strain T320A did show some tolerance to 45°C for 2 days. Additionally, DD treponeme viability in secondary culture declined with decreasing pH of the original cultures down to a pH of 5. The poor survivability of DD treponemes in these temperatures and pH values could be incorporated into DD treponeme control strategies. However, further work is required to fully understand this trend including survival studies utilising more alkaline and acidic pH values than investigated here. The importance of how multiple conditions affect the survival of DD treponemes was highlighted in the faecal microcosm survival study for the *T. medium* DD treponeme phylogroup where incubating in aerobic conditions severely inhibited survival regardless of faecal matter presence. Another study investigating *Leptospira* survival found that temperature affected the ability of the *Leptospira* species investigated to survive in different pH values. Thus future survival studies should also be conducted to better understand how the interplay between pH, temperature and oxygen concentration affects DD treponeme survival, to better inform possible control strategies that may take advantage of poor DD treponeme survival under one of these conditions.

A limitation of the survival studies carried out here was that survival was assessed for pure strains of DD treponeme phylogroups individually. A polytreponemal aetiology has been described for DD lesions (Stamm et al., 2002; Klitgaard et al., 2008; Evans et al., 2009c) and in some cases multiple phylogroups have been detected in the infection reservoirs investigated here and in other studies (Evans et al., 2012b; Klitgaard et al., 2014; Sullivan et al., 2014a, 2015a; Nascimento et al., 2015; Zinicola et al., 2015b; Angell et al., 2017). Thus it may be wise to investigate the survival of DD treponemes when multiple phylogroups or strains from the same phylogroup are incubated together under different conditions and compare with the results from individual phylogroups and strains. If survival is enhanced in a polytreponemal environment this may suggest a possible symbiosis between DD treponeme strains/ phylogroups which may partially explain why a polytreponemal aetiology in DD lesions exists.

One of the difficulties in culturing DD treponemes is that they are readily out competed by other bacterial genera present in the sample from which they are cultured from. Thus the survival of DD treponemes in the presence of other bacteria inherently present in faeces

and bedding were not investigated by sterilising the faeces and bedding. However, DD is a polymicrobial disease and unlike in culture conditions, DD treponemes are able to flourish in this polymicrobial environment. The growth and survival of DD treponemes in normal culture conditions and in different conditions such as pH, temperature etc. could be investigated with single representatives of other genera which are commonly associated with DD lesions such as *Porphymonas* spp. and *Mycoplasma* spp (Berry et al., 2010; Santos et al., 2012; Krull et al., 2014; Zinicola et al., 2015b; Nielsen et al., 2016) to determine whether individual species may actually have the ability to enhance DD treponeme survival in *in vitro* conditions and give insight into the illusive dynamics of DD as a polymicrobial disease.

7.4 Conclusions

This study has further contributed to evidence that the infection reservoirs of DD treponemes for dairy cattle are linked to the GI tract and fomites. For the first time DD treponemes have been detected on foot trimming tools other than the foot trimming knife, gloves used to handle dairy cow feet and footprints left behind on the floor. Additionally, in a first, DD treponemes have been cultivated from dairy cow faeces demonstrating that DD treponemes may be viable when detected in faeces. Survival studies demonstrated that DD treponemes have the ability to survive in sterile faeces for a median of 1 day and a maximum of 6 days depending upon phylogroup. Additionally, DD treponemes have shown the ability to remain viable in sawdust, sand and RMS whilst not being viable in straw or sand 5% (w/w) lime. DD treponemes remained viable between the temperatures of 4 and 37°C with only the *T. phagedenis* DD treponeme phylogroup demonstrating a short lived viability at 45 °C. DD treponemes were also able to remain viable between the pHs of 5.5 and 9, although optimum growth was achieved with pHs between 6.5 and 8.5 depending upon phylogroup.

The knowledge gained from this project has also raised further questions about DD infection reservoirs which will help to solidify the role of infection reservoirs in DD transmission as well as enable the identification of further reservoirs. Briefly, further investigation is required to determine the herd and management factors associated with colonisation of the GI tract and the role of the GI tract in transmission, the development of a method for diagnosing the presence of DD treponemes in the GI tract of live cattle is needed. Survival of DD treponemes in footprints and on foot trimming equipment needs to be established along with methods of disinfection and identification of other fomites which

may act as DD infection reservoirs. A survey of different bedding types for the presence of DD treponemes is also warranted. Additionally, further investigation into the survival of DD treponemes under varying oxygen concentrations would also be beneficial.

The results of this project have highlighted the importance of biosecurity for the control of DD. Although, the number and variety of possible DD infection reservoirs may make complete elimination of DD on farm very difficult. However, the knowledge gained here will further aid the dairy industry in identifying the most important infection reservoirs for DD transmission. Removing DD treponemes from infection reservoirs through disinfection or other means as well as the implementation of standard biosecurity measures and increased cleanliness should help to dramatically reduce DD on dairy farms and hopefully prevent further spread of DD amongst dairy cattle and avert cross-species transmission.

References

Agampodi, S.B., A.C. Moreno, J.M. Vinetz, and M.A. Matthias. 2013. Short report: Utility and limitations of direct multi-locus sequence typing on qPCR-positive blood to determine infecting Leptospira strain. Am. J. Trop. Med. Hyg. 88:184–185.

AHDB Dairy. 2014. Bedding Materials. Accessed September 14, 2017. https://dairy.ahdb.org.uk/technical-information/animal-health-welfare/lameness/husbandry-prevention/building-design/bedding-type/#.WbrlXsiGM2w.

AHDB Dairy. 2016a. Dairy Cow Mobility and Lameness. Accessed September 18, 2017. https://dairy.ahdb.org.uk/technical-information/animal-health-welfare/lameness/#.Wb_I58iGM2w.

AHDB Dairy. 2016b. Recycled Manure Solids. Accessed September 14, 2017. https://dairy.ahdb.org.uk/technical-information/buildings/housing/recycled-manure-solids/#.WbsErsiGM2w.

Aird, D., M.G. Ross, W.S. Chen, M. Danielsson, T. Fennell, C. Russ, D.B. Jaffe, C. Nusbaum, and A. Gnirke. 2011. Analyzing and minimizing PCR amplification bias in Illumina sequencing libraries. Genome Biol. 12:R18.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403–10.

van Amstel, S.R., S. van Vuuren, and C.L. Tutt. 1995. Digital dermatitis: report of an outbreak. J. S. Afr. Vet. Assoc. 66:177–181.

Andrucioli, M.C.D., P. Nelson-Filho, M.A.N. Matsumoto, M.C.P. Saraiva, M. Feres, L.C. De Figueiredo, and L.P. Martins. 2012. Molecular detection of in-vivo microbial contamination of metallic orthodontic brackets by checkerboard DNA-DNA hybridization. Am. J. Orthod. Dentofac. Orthop. 141:24–29.

Angell, J.W., S.R. Clegg, D.H. Grove-White, R.W. Blowey, S.D. Carter, J.S. Duncan, and N.J. Evans. 2017. Survival of contagious ovine digital dermatitis (CODD)-associated treponemes on disposable gloves after handling CODD-affected feet. Vet. Rec. 181:89.

Angell, J.W., S.R. Clegg, L.E. Sullivan, J.S. Duncan, D.H. Grove-White, S.D. Carter, and N.J. Evans. 2015a. In vitro susceptibility of contagious ovine digital dermatitis associated Treponema spp. isolates to antimicrobial agents in the UK. Vet. Dermatol. 26:484–7, e114-5.

Angell, J.W., H.E. Crosby-Durrani, J.S. Duncan, S.D. Carter, and R. Blundell. 2015b. Histopathological Characterization of the Lesions of Contagious Ovine Digital Dermatitis and Immunolabelling of Treponema-like Organisms. J. Comp. Pathol.

Angell, J.W., J.S. Duncan, S.D. Carter, and D.H. Grove-White. 2014. Farmer reported prevalence and factors associated with contagious ovine digital dermatitis in Wales: A

questionnaire of 511 sheep farmers. Prev. Vet. Med. 113:132–8.

Angell, J.W., D.H. Grove-White, and J.S. Duncan. 2018. Sheep and farm level factors associated with footrot: a longitudinal repeated cross-sectional study of sheep on six farms in the UK. Vet. Rec. 182:293.

Anklam, K., M. Kulow, W. Yamazaki, and D. Döpfer. 2017. Development of real-time PCR and loop- mediated isothermal amplification (LAMP) assays for the differential detection of digital dermatitis associated treponemes. PLoS One 12:e0178349.

Argáez-Rodríguez, F.J., D.W. Hird, J. Hernández de Anda, D.H. Read, and A. Rodríguez-Lainz. 1997. Papillomatous digital dermatitis on a commercial dairy farm in Mexicali, Mexico: incidence and effect on reproduction and milk production. Prev. Vet. Med. 32:275–86.

Ariefdjohan, M.W., D.A. Savaiano, and C.H. Nakatsu. 2010. Comparison of DNA extraction kits for PCR-DGGE analysis of human intestinal microbial communities from fecal specimens. Nutr. J. 9:23.

Barbour, A.G. 1988. Plasmid analysis of Borrelia burgdorferi, the Lyme disease agent. J. Clin. Microbiol. 26:475–478.

Bargai, U. 1994. Excessive protein as the cause of herd outbreaks of Mortellaro disease. Page 183 in Proceedings of the Eighth International symposium on Disorders of the Ruminant Digit, Banff, Canada.

Barker, Z.E., J.R. Amory, J.L. Wright, S. a Mason, R.W. Blowey, and L.E. Green. 2009. Risk factors for increased rates of sole ulcers, white line disease, and digital dermatitis in dairy cattle from twenty-seven farms in England and Wales.. J. Dairy Sci. 92:1971–1978.

Barker, Z.E., K.A. Leach, H.R. Whay, N.J. Bell, and D.C.J. Main. 2010. Assessment of lameness prevalence and associated risk factors in dairy herds in England and Wales. J. Dairy Sci. 93:932–41.

Bassett, H.F., M.L. Monaghan, P. Lenhan, M.L. Doherty, and M.E. Carter. 1990. Bovine digital dermatitis. Vet. Rec. 126:164–5.

Becker, P.S., D.R. Akins, J.D. Radolf, and M. V Norgard. 1994. Similarity between the 38-kilodalton lipoprotein of Treponema pallidum and the glucose/galactose-binding (MglB) protein of Escherichia coli. Infect. Immun. 62:1381–91.

Bekele, A.Z., S. Koike, and Y. Kobayashi. 2011. Phylogenetic diversity and dietary association of rumen Treponema revealed using group-specific 16S rRNA gene-based analysis. FEMS Microbiol. Lett. 316:51–60.

Benson, D.A., M. Cavanaugh, K. Clark, I. Karsch-Mizrachi, D.J. Lipman, J. Ostell, and E.W. Sayers. 2013. GenBank. Nucleic Acids Res. 41:D36–D42.

Berry, S.L., D.H. Read, T.R. Famula, A. Mongini, and D. Döpfer. 2012. Long-term observations on the dynamics of bovine digital dermatitis lesions on a California dairy after topical treatment with lincomycin HCl. Vet. J. 193:654–658.

Berry, S.L., D.H. Read, R.L. Walker, and T.R. Famula. 2010. Clinical, histologic, and bacteriologic findings in dairy cows with digital dermatitis (footwarts) one month after

topical treatment with lincomycin hydrochloride or oxytetracycline hydrochloride. J. Am. Vet. Med. Assoc. 237:555–560.

Bizzini, A., and G. Greub. 2010. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry, a revolution in clinical microbial identification. Clin. Microbiol. Infect. 16:1614–1619.

Blowey, R. 2005. Factors associated with lameness in dairy cattle. In Pract. 27:154–162.

Blowey, R., and M. Sharp. 1988. Digital dermatitis in dairy cattle. Vet. Rec. 122:505–508.

Blowey, R.W., S.D. Carter, A.G. White, and A. Barnes. 1994a. Borrelia burgdorferi infections in UK cattle: a possible association with digital dermatitis. Vet. Rec. 135:577-578; 16 .

Blowey, R.W., S.H. Done, and W. Cooley. 1994b. Observations on the pathogenesis of digital dermatitis in cattle. Vet. Rec. 135:115–117.

Borsanelli, A.C., E. Gaetti-Jardim Júnior, J. Döbereiner, and I.S. Dutra. 2015. Treponema denticola in microflora of bovine periodontitis. Pesqui. Veterinária Bras. 35:237–240.

Borsanelli, A.C., T.N.M. Ramos, E. Gaetti-Jardim, and C.M. Schweitzer. 2017. Treponema species in the subgingival microflora of ovine periodontitis. Vet. Rec. 180:150.

Boye, M., S.B. Baloda, T.D. Leser, and K. Møller. 2001. Survival of Brachyspira hyodysenteriae and B. pilosicoli in terrestrial microcosms. Vet. Microbiol. 81:33–40.

Bradley, A., K. Leach, C. Ohnstad, M. Green, V. Prout, B. Payne, and D. Black. 2016. Preliminary Results of an Investigation of the Impact of Bedding Type on Pathogen Load in Bedding, Udder Health and Milk Quality. Page 177 in Proceedings of the World Buiatrics Congress 2016, Dublin, Ireland.

Brenner, D.J., N.R. Krieg, and J.T. Staley. 1984. Bergeys Manual of Systematic Bacteriology. Volume 1. Williams and Wilkins, Baltimore/ London.

Britt, J.S., J. Gaska, E.F. Garrett, D. Konkle, and M. Mealy. 1996. Comparison of topical application of three products for treatment of papillomatous digital dermatitis in dairy cattle. J. Am. Vet. Med. Assoc. 209:1134–1136.

Brorson, and S.H. Brorson. 1997. Transformation of cystic forms of Borrelia burgdorferi to normal, mobile spirochetes. Infection 25:240–246.

Brown, C.C., P.D. Kilgo, and K.L. Jacobsen. 2000. Prevalence of papillomatous digital dermatitis among culled adult cattle in the southeastern United States. Am. J. Vet. Res. 61:928–930.

Bruijnis, M.R.N., B. Beerda, H. Hogeveen, and E.N. Stassen. 2012. Assessing the welfare impact of foot disorders in dairy cattle by a modeling approach. animal 6:962–970.

Bruijnis, M.R.N., H. Hogeveen, and E.N. Stassen. 2010. Assessing economic consequences of foot disorders in dairy cattle using a dynamic stochastic simulation model. J. Dairy Sci. 93:2419–2432.

Budras, K., H. König, R. Habel, C. Muilling, P. Greenough, A. Wunsche, and S. Buda. 2003. Dermis of the Hoof. Bovine Anatomy. 2nd Ed. Schlütersche, Germany.

Calderaro, A., G. Piccolo, S. Montecchini, M. Buttrini, C. Gorrini, S. Rossi, M.C. Arcangeletti,

F. De Conto, M.C. Medici, and C. Chezzi. 2013. MALDI-TOF MS analysis of human and animal Brachyspira species and benefits of database extension. J. Proteomics 78:273–280.

Canale-Parola, E. 1977. Physiology and evolution of spirochetes. Bacteriol. Rev. 41:181–204.

Canale-Parola, E. 1978. Motility and Chemotaxis of Spirochetes. Annu. Rev. Microbiol. 32:69–97.

Capion, N., M. Boye, C. Ekstrøm, K. Dupont, and T.K. Jensen. 2013. A Study into Digital Dermatitis Transmission and Bacterial Associated Pathological Changes Involved in the Disease. Open J. Vet. Med. 3:192–198.

Capion, N., S.M. Thamsborg, and C. Enevoldsen. 2009. Prevalence and severity of foot lesions in Danish Holstein heifers through first lactation. Vet. J. 182:50–58.

Carozzi, F.M., and C. Sani. 2013. Fecal Collection and Stabilization Methods for Improved Fecal DNA Test for Colorectal Cancer in a Screening Setting. J. Cancer Res. 2013:1–8.

Cha, E., J.A. Hertl, D. Bar, and Y.T. Gröhn. 2010. The cost of different types of lameness in dairy cows calculated by dynamic programming. Prev. Vet. Med. 97:1–8.

Chapman, P.A., D.J. Wright, and P. Norman. 1989. Verotoxin-producing Escherichia coli infections in Sheffield: Cattle as a possible source. Epidemiol. Infect. 102:439–445.

Charon, N.W., and S.F. Goldstein. 2002. Genetics of Motility and Chemotaxis of a Fascinating Group of Bacteria: The Spirochetes. Annu. Rev. Genet. 36:47–73.

Cheli, R., and C. Mortellaro. 1974. Digital dermatitis in dairy cattle. Pages 208–213 in Proceedings of the 8th international conference of diseases of cattle, Milan, Italy.

Chen, H., M. Rangasamy, S.Y. Tan, H. Wang, and B.D. Siegfried. 2010. Evaluation of five methods for total DNA extraction from western corn rootworm beetles. PLoS One 5:e11963.

Choi, B.K., H. Nattermann, S. Grund, W. Haider, and U.B. Göbel. 1997. Spirochetes from digital dermatitis lesions in cattle are closely related to treponemes associated with human periodontitis. Int. J. Syst. Bacteriol. 47:175–81.

Choi, B.K., B.J. Paster, F.E. Dewhirst, and U.B. Gobel. 1994. Diversity of cultivable and uncultivable oral spirochetes from a patient with severe destructive periodontitis. Infect. Immun. 62:1889–1895.

Chu, L., W. Kennell, and S.C. Holt. 1994. Characterization of hemolysis and hemoxidation activities by Treponema denticola. Microb. Pathog. 16:183–95.

Chua, P.K.B., J.E. Corkill, P.S. Hooi, S.C. Cheng, C. Winstanley, and C.A. Hart. 2005. Isolation of Waddlia malaysiensis, a novel intracellular bacterium, from fruit bat (Eonycteris spelaea). Emerg. Infect. Dis. 11:271–7.

Claassen, S., E. du Toit, M. Kaba, C. Moodley, H.J. Zar, and M.P. Nicol. 2013. A comparison of the efficiency of five different commercial DNA extraction kits for extraction of DNA from faecal samples. J. Microbiol. Methods 94:103–10.

Clegg, S.R., J. Bell, S. Ainsworth, R.W. Blowey, N.J. Bell, S.D. Carter, and N.J. Evans. 2016a.

Isolation of digital dermatitis treponemes from cattle hock skin lesions. Vet. Dermatol.

Clegg, S.R., S.D. Carter, R.J. Birtles, J.M. Brown, C.A. Hart, and N.J. Evans. 2016b. Multi locus sequence typing of pathogenic treponemes isolated from cloven hoofed animals and their comparison to human isolates. Appl. Environ. Microbiol. AEM.00025-16-.

Clegg, S.R., S.D. Carter, J.P. Stewart, D.M. Amin, R.W. Blowey, and N.J. Evans. 2016c. Bovine ischaemic teat necrosis: a further potential role for digital dermatitis treponemes. Vet. Rec. vetrec-2015-103167.

Clegg, S.R., K.P. Coyne, J. Parker, S. Dawson, S.A. Godsall, G. Pinchbeck, P.J. Cripps, R.M. Gaskell, and A.D. Radford. 2011. Molecular epidemiology and phylogeny reveal complex spatial dynamics in areas where canine parvovirus is endemic. J. Virol. 85:7892–9.

Clegg, S.R., H.E. Crosby-Durrani, J. Bell, R. Blundell, R.W. Blowey, S.D. Carter, and N.J. Evans. 2016d. Detection and Isolation of Digital Dermatitis Treponemes from Bovine Pressure Sores. J. Comp. Pathol.

Clegg, S.R., K.G. Mansfield, K. Newbrook, L.E. Sullivan, R.W. Blowey, S.D. Carter, and N.J. Evans. 2015. Isolation of digital dermatitis treponemes from hoof lesions in Wild North American Elk (Cervus elaphus) in Washington State, USA. J. Clin. Microbiol. 53:88–94.

Clegg, S.R., L.E. Sullivan, J. Bell, R.W. Blowey, S.D. Carter, and N.J. Evans. 2016e. Detection and isolation of digital dermatitis treponemes from skin and tail lesions in pigs. Res. Vet. Sci. 104:64–70.

Clopper, C.J., and E.S. Pearson. 1934. The Use of Confidence or Fiducial Limits Illustrated in the Case of the Binomial. Biometrika 26:404.

Cook, K.L., and J.S. Britt. 2007. Optimization of methods for detecting Mycobacterium avium subsp. paratuberculosis in environmental samples using quantitative, real-time PCR. J. Microbiol. Methods 69:154–160.

Cook, N.B. 2003. Prevalence of lameness among dairy cattle in Wisconsin as a function of housing type and stall surface. J. Am. Vet. Med. Assoc. 223:1324–1328.

Cornelisse, J.L., D.J. Peterse, and E. Toussaint Raven. 1981. [A digital disorder in dairy cattle. Dermatitis digitalis? (author's transl)]. Tijdschr Diergeneeskd 106:452–455.

Cox, A., P. Dixon, and S. Smith. 2012. Histopathological lesions associated with equine periodontal disease. Vet. J. 194:386–391.

Cramer, G., K.D. Lissemore, C.L. Guard, K.E. Leslie, and D.F. Kelton. 2009. Herd-level risk factors for seven different foot lesions in Ontario Holstein cattle housed in tie stalls or free stalls. J. Dairy Sci. 92:1404–11.

Crosby-Durrani, H.E., S.R. Clegg, E. Singer, J.W. Angell, N.J. Evans, S.D. Carter, R.J. Blundell, and J.S. Duncan. 2016. Severe Foot Lesions in Dairy Goats Associated with Digital Dermatitis Treponemes. J. Comp. Pathol.

Crossley, B.M., F.J. Zagmutt-Vergara, T.L. Fyock, R.H. Whitlock, and I.A. Gardner. 2005. Fecal shedding of Mycobacterium avium subsp. paratuberculosis by dairy cows. Vet. Microbiol. 107:257–263.

Cruz, C., D. Driemeier, C. Cerva, and L.G. Corbellini. 2001. Bovine digital dermatitis in southern Brazil. Vet. Rec. 148:576–577.

Cruz, C.E.F., C.A. Pescador, Y. Nakajima, and D. Driemeier. 2005. Immunopathological investigations on bovine digital epidermitis. Vet. Rec. 157:834–840.

Danner, H., M. Holzer, E. Mayrhuber, and R. Braun. 2003. Acetic acid increases stability of silage under aerobic conditions. Appl. Environ. Microbiol. 69:562–567.

Dashper, S.G., C.A. Seers, K.H. Tan, and E.C. Reynolds. 2011. Virulence factors of the oral spirochete Treponema denticola. J. Dent. Res. 90:691–703.

Davies, I.H., R.D. Naylor, and P.K. Martin. 1999. Severe ovine foot disease. Vet. Rec. 145:646.

Davies, P.L., J.A. Leigh, A.J. Bradley, S.C. Archer, R.D. Emes, and M.J. Green. 2016. Molecular Epidemiology of Streptococcus uberis Clinical Mastitis in Dairy Herds: Strain Heterogeneity and Transmission. J. Clin. Microbiol. 54:68–74.

Demirkan, I., S.D. Carter, R.D. Murray, R.W. Blowey, and M.J. Woodward. 1998. The frequent detection of a treponeme in bovine digital dermatitis by immunocytochemistry and polymerase chain reaction. Vet. Microbiol. 60:285–292.

Demirkan, I., R.L. Walker, R.D. Murray, R.W. Blowey, and S.D. Carter. 1999. Serological evidence of spirochaetal infections associated with digital dermatitis in dairy cattle. Vet. J. 157:69–77.

Desneux, J., and A.-M. Pourcher. 2014. Comparison of DNA extraction kits and modification of DNA elution procedure for the quantitation of subdominant bacteria from piggery effluents with real-time PCR. Microbiologyopen 3:437–45.

DeVries, T.J., S. Dufour, and D.T. Scholl. 2010. Relationship between feeding strategy, lying behavior patterns, and incidence of intramammary infection in dairy cows. J. Dairy Sci. 93:1987–97.

Dewhirst, F.E., M.A. Tamer, R.E. Ericson, C.N. Lau, V.A. Levanos, S.K. Boches, J.L. Galvin, and B.J. Paster. 2000. The diversity of periodontal spirochetes by 16S rRNA analysis. Oral Microbiol. Immunol. 15:196–202.

Dhawi, A., C.A. Hart, I. Demirkan, I.H. Davies, and S.D. Carter. 2005. Bovine digital dermatitis and severe virulent ovine foot rot: a common spirochaetal pathogenesis. Vet. J. 169:232–41.

Dopfer, D. 2009. Digital dermatitis. The dynamics of digital dermatitis in dairy cattle and the manageable state of disease. Pages 1–5 in CanWest Veterinary Conference, Banff, Canada.

Döpfer, D., K. Anklam, D. Mikheil, and P. Ladell. 2012a. Growth curves and morphology of three Treponema subtypes isolated from digital dermatitis in cattle. Vet. J. 193:685–93.

Döpfer, D., M. Holzhauer, and M. van Boven. 2012b. The dynamics of digital dermatitis in populations of dairy cattle: Model-based estimates of transition rates and implications for control. Vet. J. 193:648–653.

Dopfer, D., A.A.H.M. ter Huurne, J.L. Cornelisse, A.J.A.M. van Asten, A. Koopmans, F.A. Meijer, Y.H. Schukken, I. Szakall, W. Klee, and R.B. Bosma. 1997. Histological and bacteriological evaluation of digital dermatitis in cattle, with special reference to spirochaetes and Campylobacter faecalis. Vet. Rec. 140:620–623.

Duff, J.W., J.S. Pittman, D. Abvp, J.M. Hammer, J.M. Kinyon, and R. Prevalencia. 2014. Prevalence of Brachyspira hyodysenteriae in sows and suckling piglets. J. Swine Heal. Prod. 22:71–77.

Dunn, O.J. 1961. Multiple Comparisons among Means. J. Am. Stat. Assoc. 56:52–64.

Dyer, R.M., N.K. Neerchal, U. Tasch, Y. Wu, P. Dyer, and P.G. Rajkondawar. 2007. Objective determination of claw pain and its relationship to limb locomotion score in dairy cattle. J. Dairy Sci. 90:4592–602.

Edwards, A.M., D. Dymock, M.J. Woodward, and H.F. Jenkinson. 2003a. Genetic relatedness and phenotypic characteristics of Treponema associated with human periodontal tissues and ruminant foot disease. Microbiology 149:1083–1093.

Edwards, A.M.M., D. Dymock, and H.F.F. Jenkinson. 2003b. From tooth to hoof: treponemes in tissue-destructive diseases. J. Appl. Microbiol. 94:767–780.

Ehresmann, C., P. Stiegler, G.A. Mackie, R.A. Zimmermann, J.P. Ebel, and P. Fellner. 1975. Primary sequence of the 16S ribosomal RNA of Escherichia coli. Nucleic Acids Res. 2:265–278.

Eicher, S.D., D.C. Lay, J.D. Arthington, and M.M. Schutz. 2013. Effects of rubber flooring during the first 2 lactations on production, locomotion, hoof health, immune functions, and stress. J. Dairy Sci. 96:3639–3651.

el-Ghoul, W., and B.I. Shaheed. 2001. Ulcerative and papillomatous digital dermatitis of the pastern region in dairy cattle: clinical and histopathological studies. Dtsch. Tierarztl. Wochenschr. 108:216–222.

Enevoldsen, C., Y.T. Gröhn, and I. Thysen. 1994. Skin injuries on the body and thigh of dairy cows: associations with season, claw health, disease treatment, and other cow characteristics.. Acta Vet. Scand. 35:337–47.

Evans, N.J., R.W. Blowey, D. Timofte, D.R. Isherwood, J.M. Brown, R. Murray, R.J. Paton, and S.D. Carter. 2011a. Association between bovine digital dermatitis treponemes and a range of “non-healing” bovine hoof disorders. Vet. Rec. 168:214.

Evans, N.J., J.M. Brown, I. Demirkan, R. Birtles, C.A. Hart, and S.D. Carter. 2009a. In vitro susceptibility of bovine digital dermatitis associated spirochaetes to antimicrobial agents. Vet. Microbiol. 136:115–20.

Evans, N.J., J.M. Brown, I. Demirkan, R.D. Murray, R.J. Birtles, C.A. Hart, and S.D. Carter. 2009b. Treponema pedis sp. nov., a spirochaete isolated from bovine digital dermatitis lesions. Int. J. Syst. Evol. Microbiol. 59:987–91.

Evans, N.J., J.M. Brown, I. Demirkan, R.D. Murray, W.D. Vink, R.W. Blowey, C.A. Hart, and S.D. Carter. 2008. Three unique groups of spirochetes isolated from digital dermatitis lesions in UK cattle. Vet. Microbiol. 130:141–50.

Evans, N.J., J.M. Brown, I. Demirkan, P. Singh, B. Getty, D. Timofte, W.D. Vink, R.D. Murray,

R.W. Blowey, R.J. Birtles, C.A. Hart, and S.D. Carter. 2009c. Association of unique, isolated treponemes with bovine digital dermatitis lesions. J. Clin. Microbiol. 47:689–96.

Evans, N.J., J.M. Brown, C. Hartley, R.F. Smith, and S.D. Carter. 2012a. Antimicrobial susceptibility testing of bovine digital dermatitis treponemes identifies macrolides for in vivo efficacy testing. Vet. Microbiol. 160:496–500.

Evans, N.J., J.M. Brown, R.D. Murray, B. Getty, R.J. Birtles, C.A. Hart, and S.D. Carter. 2011b. Characterization of novel bovine gastrointestinal tract Treponema isolates and comparison with bovine digital dermatitis treponemes. Appl. Environ. Microbiol. 77:138–47.

Evans, N.J., R.D. Murray, and S.D. Carter. 2016. Bovine digital dermatitis: current concepts from laboratory to farm. Vet. J.

Evans, N.J., D. Timofte, S.D. Carter, J.M. Brown, R. Scholey, D.H. Read, and R.W. Blowey. 2010. Association of treponemes with bovine ulcerative mammary dermatitis. Vet. Rec. 166:532–533.

Evans, N.J., D. Timofte, D.R. Isherwood, J.M. Brown, J.M. Williams, K. Sherlock, M.J. Lehane, R.D. Murray, R.J. Birtles, C.A. Hart, and S.D. Carter. 2012b. Host and environmental reservoirs of infection for bovine digital dermatitis treponemes. Vet. Microbiol. 156:102–9.

Fernando, W.L. 1969. Erythromycin in early syphilis.. Br. J. Vener. Dis. 45:200–1.

Ferrand, J., K. Patron, C. Legrand-Frossi, J.-P. Frippiat, C. Merlin, C. Alauzet, and A. Lozniewski. 2014. Comparison of seven methods for extraction of bacterial DNA from fecal and cecal samples of mice. J. Microbiol. Methods 105:180–5.

Fisher, R.A. 1922. On the Interpretation of χ 2 from Contingency Tables, and the Calculation of P. J. R. Stat. Soc. 85:87.

Fjeldaas, T., Å.M. Sogstad, and O. Østerås. 2011. Locomotion and claw disorders in Norwegian dairy cows housed in freestalls with slatted concrete, solid concrete, or solid rubber flooring in the alleys. J. Dairy Sci. 94:1243–1255.

Frankena, K., J.G.C.J. Somers, W.G.P. Schouten, J. V van Stek, J.H.M. Metz, E.N. Stassen, and E.A.M. Graat. 2009. The effect of digital lesions and floor type on locomotion score in Dutch dairy cows. Prev. Vet. Med. 88:150–7.

Gao, W., Y. Chan, M. You, D.C. Lacap-Bugler, W.K. Leung, and R.M. Watt. 2015. In-depth snapshot of the equine subgingival microbiome. Microb. Pathog.

GB Cattle Health and Welfare Group. 2014. Second Annual Report - GB Cattle Health and Welfare Group, 2014.

Godden, S., R. Bey, K. Lorch, R. Farnsworth, P. Rapnicki, W.D. Hueston, D.E. Pritchard, G.L. Bowman, L.E. Heider, B.L. Brockett, and H.R. Conrad. 2008. Ability of organic and inorganic bedding materials to promote growth of environmental bacteria. J. Dairy Sci. 91:151–159.

Gomez, A., K.S. Anklam, N.B. Cook, J. Rieman, K.A. Dunbar, K.E. Cooley, M.T. Socha, and D. Döpfer. 2014a. Immune response against Treponema spp. and ELISA detection of

digital dermatitis. J. Dairy Sci. 97:4864–75.

Gomez, A., N. Bernardoni, J. Rieman, A. Dusick, R. Hartshorn, D.H. Read, M.T. Socha, N.B. Cook, and D. Döpfer. 2014b. A randomized trial to evaluate the effect of a trace mineral premix on the incidence of active digital dermatitis lesions in cattle. J. Dairy Sci. 97:6211–22.

Gomez, A., N.B. Cook, N.D. Bernardoni, J. Rieman, A.F. Dusick, R. Hartshorn, M.T. Socha, D.H. Read, and D. Döpfer. 2012. An experimental infection model to induce digital dermatitis infection in cattle. J. Dairy Sci. 95:1821–30.

González, L.A., B.J. Tolkamp, M.P. Coffey, A. Ferret, and I. Kyriazakis. 2008. Changes in feeding behavior as possible indicators for the automatic monitoring of health disorders in dairy cows. J. Dairy Sci. 91:1017–28.

Graber, J.R., J.R. Leadbetter, and J.A. Breznak. 2004. Description of Treponema azotonutricium sp. nov. and Treponema primitia sp. nov., the First Spirochetes Isolated from Termite Guts. Appl. Environ. Microbiol. 70:1315–1320.

Gressley, T.F., M.B. Hall, and L.E. Armentano. 2011. Ruminant Nutrition Symposium: Productivity, digestion, and health responses to hindgut acidosis in ruminants. J. Anim. Sci. 89:1120–30.

Harry, M., B. Gambier, Y. Bourezgui, and E. Garnier-Sillam. 1999. Evaluation of purification procedures for DNA extracted from rich organic samples: interference with humic substances. Analusis 27:439–441.

Hart, M.L., A. Meyer, P.J. Johnson, A.C. Ericsson, D. Savage, R. Ley, M. Hamady, C. Lozupone, P. Turnbaugh, R. Ramey, J. Bircher, F. Martin, M. Dumas, Y. Wang, C. Legido-Quigley, I. Yap, H. Tang, H. Chung, S. Pamp, J. Hill, N. Surana, S. Edelman, E. Troy, L. Hooper, D. Littman, A. Macpherson, W. Strober, J. Kinross, A. Darzi, J. Nicholson, I. Sekirov, S. Russell, L. Antunes, B. Finlay, P. Hugenholtz, G. Henderson, F. Cox, S. Kittelmann, V. Miri, M. Zethof, S. Noel, M. Li, J. Gong, M. Cottrill, H. Yu, C. de Lange, J. Burton, J. Ferrand, K. Patron, C. Legrand-Frossi, J. Frippiat, C. Merlin, C. Alauzet, N. Kennedy, A. Walker, S. Berry, S. Duncan, F. Farquarson, P. Louis, A. Wesolowska-Andersen, M. Bahl, V. Carvalho, K. Kristiansen, T. Sicheritz-Ponten, R. Gupta, F. Guo, T. Zhang, S. Yuan, D. Cohen, J. Ravel, Z. Abdo, L. Forney, G. Wu, J. Lewis, C. Hoffmann, Y. Chen, R. Knight, K. Bittinger, A. Ericsson, J. Davis, W. Spollen, N. Bivens, S. Givan, C. Hagan, T. Magoc, S. Salzberg, J. Kuczynski, J. Stombaugh, W. Walters, A. Gonzalez, J. Caporaso, R. Knight, S. Altschul, T. Madden, A. Schaffer, J. Zhang, Z. Zhang, W. Miller, T. DeSantis, P. Hugenholtz, N. Larsen, M. Rojas, E. Brodie, K. Keller, G. Roeselers, E. Mittge, W. Stephens, D. Parichy, C. Cavanaugh, K. Guillemin, F. Collins, and L. Tabak. 2015. Comparative Evaluation of DNA Extraction Methods from Feces of Multiple Host Species for Downstream Next-Generation Sequencing. PLoS One 10:e0143334.

Hartshorn, R.E.E., E.C.C. Thomas, K. Anklam, M.G.G. Lopez-Benavides, M. Buchalova, T.C.C. Hemling, and D. Döpfer. 2013. Short communication: minimum bactericidal concentration of disinfectants evaluated for bovine digital dermatitis-associated Treponema phagedenis-like spirochetes. J. Dairy Sci. 96:3034–8.

Harwood, D.G., J.H. Cattell, C.J. Lewis, and R. Naylor. 1997. Virulent foot rot in sheep. Vet. Rec. 140:687.

Hasan, A., and R.M. Palmer. 2014. A clinical guide to periodontology: Pathology of periodontal disease. Br. Dent. J. 216:457–461.

Hernandez, J., J.K. Shearer, and J.B. Elliott. 1999. Comparison of topical application of oxytetracycline and four nonantibiotic solutions for treatment of papillomatous digital dermatitis in dairy cows. J. Am. Vet. Med. Assoc. 214:688–690.

Hernandez, J., J.K. Shearer, and D.W. Webb. 2001. Effect of lameness on the calving-to-conception interval in dairy cows. J. Am. Vet. Med. Assoc. 218:1611–4.

Hernandez, J., J.K. Shearer, and D.W. Webb. 2002. Effect of lameness on milk yield in dairy cows. J. Am. Vet. Med. Assoc. 220:640–4.

Hogan, J.S., V.L. Bogacz, L.M. Thompson, S. Romig, P.S. Schoenberger, W.P. Weiss, and K.L. Smith. 1999. Bacterial Counts Associated with Sawdust and Recycled Manure Bedding Treated with Commercial Conditioners. J. Dairy Sci. 82:1690–1695.

Hogan, J.S., and K.L. Smith. 1997. Bacteria counts in sawdust bedding. J. Dairy Sci. 80:1600–1605.

Hogan, J.S., K.L. Smith, K.H. Hoblet, D.A. Todhunter, P.S. Schoenberger, W.D. Hueston, D.E. Pritchard, G.L. Bowman, L.E. Heider, and B.L. Brockett. 1989. Bacterial counts in bedding materials used on nine commercial dairies. J. Dairy Sci. 72:250–8.

Holloway, J.W., R.E.I. Estell, and W.T.J. Butts. 1981. Relationship between Fecal Components and Forage Consumption and Digestibility. J. Anim. Sci. 52:836–848.

Holzhauer, M., C.J. Bartels, C. Bergsten, M.M.J. van Riet, K. Frankena, and T.J.G.M. Lam. 2012. The effect of an acidified, ionized copper sulphate solution on digital dermatitis in dairy cows. Vet. J. 193:659–663.

Holzhauer, M., C. Hardenberg, C.J.M. Bartels, and K. Frankena. 2006. Herd- and cow-level prevalence of digital dermatitis in the Netherlands and associated risk factors. J. Dairy Sci. 89:580–8.

Houston, S., J.S. Taylor, Y. Denchev, R. Hof, R.L. Zuerner, and C.E. Cameron. 2015. Conservation of the Host-Interacting Proteins Tp0750 and Pallilysin among Treponemes and Restriction of Proteolytic Capacity to Treponema pallidum. Infect. Immun. 83:4204–16.

Hultgren, J., and C. Bergsten. 2001. Effects of a rubber-slatted flooring system on cleanliness and foot health in tied dairy cows. Prev. Vet. Med. 52:75–89.

Huson, D.H., and D. Bryant. 2005. Application of Phylogenetic Networks in Evolutionary Studies. Mol. Biol. Evol. 23:254–267.

Idsoe, O., T. Guthe, and R.R. Willcox. 1972. Penicillin in the treatment of syphilis. The experience of three decades.. Bull. World Health Organ. 47 Suppl:1–68.

Inglis, G.D., and L.D. Kalischuk. 2003. Use of PCR for direct detection of Campylobacter species in bovine feces. Appl. Environ. Microbiol. 69:3435–47.

Inglis, G.D., L.D. Kalischuk, and H.W. Busz. 2004. Chronic shedding of Campylobacter species in beef cattle. J. Appl. Microbiol. 97:410–420.

Ito, K., M.A.G. von Keyserlingk, S.J. Leblanc, and D.M. Weary. 2010. Lying behavior as an

indicator of lameness in dairy cows. J. Dairy Sci. 93:3553–60.

Jelocnik, M., E. Walker, Y. Pannekoek, J. Ellem, P. Timms, and A. Polkinghorne. 2014. Evaluation of the relationship between Chlamydia pecorum sequence types and disease using a species-specific multi-locus sequence typing scheme (MLST). Vet. Microbiol. 174:214–222.

Joseph, R., S.C. Holt, and E. Canale-Parola. 1973. Peptidoglycan of free-living anaerobic spirochetes. J. Bacteriol. 115:426–35.

Justice-Allen, A., J. Trujillo, R. Corbett, R. Harding, G. Goodell, D. Wilson, and M. Sueyoshi. 2010. Survival and replication of Mycoplasma species in recycled bedding sand and association with mastitis on dairy farms in Utah. J. Dairy Sci. 93:192–202.

Karlsson, F., K. Klitgaard, and T.K. Jensen. 2014. Identification of Treponema pedis as the predominant Treponema species in porcine skin ulcers by fluorescence in situ hybridization and high-throughput sequencing. Vet. Microbiol. 171:122–131.

Keen, J.E., W.W. Laegreid, C.G. Chitko-McKown, L.M. Durso, and J.L. Bono. 2010. Distribution of Shiga-toxigenic Escherichia coli O157 in the gastrointestinal tract of naturally O157-shedding cattle at necropsy. Appl. Environ. Microbiol. 76:5278–81.

Keys, J.E., L.W. Smith, B.T. Weinland, and D.L. Hill. 1976. Response of Dairy Cattle Given a Free Choice of Free Stall Location and Three Bedding Materials. J. Dairy Sci. 59:1157–1162.

Kimura, Y., M. Takahashi, N. Matsumoto, H. Tsukida, M. Satoh, K. Ohkawara, M. Kanoe, N. Gotoh, M. Kubo, O. Aoki, and M. Hataya. 1993. Verrucose dermatitis and digital papillomatosis in dairy cows. J. Vet. Med. Japan 46:899–906.

Kleen, J.L., L. Upgang, and J. Rehage. 2013. Prevalence and consequences of subacute ruminal acidosis in German dairy herds. Acta Vet. Scand. 55:48.

Klitgaard, K., M. Boye, N. Capion, and T.K. Jensen. 2008. Evidence of multiple Treponema phylotypes involved in bovine digital dermatitis as shown by 16S rRNA gene analysis and fluorescence in situ hybridization. J. Clin. Microbiol. 46:3012–20.

Klitgaard, K., A.F. Bretó, M. Boye, and T.K. Jensen. 2013. Targeting the treponemal microbiome of digital dermatitis infections by high-resolution phylogenetic analyses and comparison with fluorescent in situ hybridization. J. Clin. Microbiol. 51:2212–2219.

Klitgaard, K., M.W. Nielsen, H.-C. Ingerslev, M. Boye, and T.K. Jensen. 2014. Discovery of Bovine Digital Dermatitis-Associated Treponema spp. in the Dairy Herd Environment by a Targeted Deep-Sequencing Approach. Appl. Environ. Microbiol. 80:4427–32.

Klitgaard, K., M.L. Strube, A. Isbrand, T.K. Jensen, and M.W. Nielsen. 2017. Microbiota analysis of an environmental slurry and its potential role as a reservoir of bovine digital dermatitis pathogens. Appl. Environ. Microbiol. 83:AEM.00244-17.

Knappe-Poindecker, M., M. Gilhuus, T.K. Jensen, K. Klitgaard, R.B. Larssen, and T. Fjeldaas. 2013. Interdigital dermatitis, heel horn erosion, and digital dermatitis in 14 Norwegian dairy herds. J. Dairy Sci. 96:7617–29.

Koenig, S., A.R. Sharifi, H. Wentrot, D. Landmann, M. Eise, and H. Simianer. 2005. Genetic

Parameters of Claw and Foot Disorders Estimated with Logistic Models. J. Dairy Sci. 88:3316–3325.

Kofoed, K., J. Gerstoft, L.R. Mathiesen, and T. Benfield. 2006. Syphilis and Human Immunodeficiency Virus (HIV)-1 Coinfection: Influence on CD4 T-Cell Count, HIV-1 Viral Load, and Treatment Response. Sex. Transm. Dis. 33:143–148.

Koniarová, I., A. Orsag, and V. Ledecký. 1993. [The role anaerobes in dermatitis digitalis et interdigitalis in cattle]. Vet. Med. (Praha). 38:589–96.

Krause, K.M., and G.R. Oetzel. 2005. Inducing Subacute Ruminal Acidosis in Lactating Dairy Cows. J. Dairy Sci. 88:3633–3639.

Kreader, C.A. 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Appl. Environ. Microbiol. 62:1102–6.

Krull, A.C., V.L. Cooper, J.W. Coatney, J.K. Shearer, P.J. Gorden, and P.J. Plummer. 2016. A Highly Effective Protocol for the Rapid and Consistent Induction of Digital Dermatitis in Holstein Calves. PLoS One 11:e0154481.

Krull, A.C., J.K. Shearer, P.J. Gorden, V.L. Cooper, G.J. Phillips, and P.J. Plummer. 2014. Deep Sequencing Analysis Reveals the Temporal Microbiota Changes Associated with the Development of Bovine Digital Dermatitis. Infect. Immun. IAI.02077-14-.

Kuoppa, Y., J. Boman, L. Scott, U. Kumlin, I. Eriksson, and A. Allard. 2002. Quantitative detection of respiratory Chlamydia pneumoniae infection by real-time PCR. J. Clin. Microbiol. 40:2273–4.

Langrell, S.R.H. 2005. Development of a nested PCR detection procedure for Nectria fuckeliana direct from Norway spruce bark extracts. FEMS Microbiol. Lett. 242:185–193.

Laven, R. 2001. Control of digital dermatitis in cattle. In Pract. 23:336–341.

Laven, R.A. 1999. The environment and digital dermatitis. Cattle Pract. 7:349–354.

Laven, R.A. 2006. Efficacy of systemic cefquinome and erythromycin against digital dermatitis in cattle. Vet. Rec. 159:19–20.

Laven, R.A., K.R. Lawrence, E. Burrough, A.J. Webster, J.W. Hughes, F.J. Manson, J.B. Merritt, W.B. Russell, J.E. Sutherst, and W.R. Ward. 2006. An evaluation of the seasonality of veterinary treatments for lameness in UK dairy cattle. J. Dairy Sci. 89:3858–65.

Laven, R.A., and D.N. Logue. 2006. Treatment strategies for digital dermatitis for the UK. Vet. J. 171:79–88.

Laven, R.A., and M.J. Proven. 2000. Use of an antibiotic footbath in the treatment of bovine digital dermatitis. Vet. Rec. 147:503–6.

Leach, K.A., S.C. Archer, J.E. Breen, M.J. Green, I.C. Ohnstad, S. Tuer, and A.J. Bradley. 2015. Recycling manure as cow bedding: Potential benefits and risks for UK dairy farms. Vet. J. 206:123–130.

Lean, I.J., C.T. Westwood, H.M. Golder, and J.J. Vermunt. 2013. Impact of nutrition on lameness and claw health in cattle. Livest. Sci. 156:71–87.

Lee, S.H., V.S. Vigliotti, J.S. Vigliotti, W. Jones, J. Williams, and J. Walshon. 2010. Early Lyme disease with spirochetemia - diagnosed by DNA sequencing. BMC Res. Notes 3:273.

LeJeune, J.T., and M.D. Kauffman. 2005. Effect of Sand and Sawdust Bedding Materials on the Fecal Prevalence of Escherichia coli O157:H7 in Dairy Cows. Appl. Environ. Microbiol. 71:326–330.

Li, M., J. Gong, M. Cottrill, H. Yu, C. de Lange, J. Burton, and E. Topp. 2003. Evaluation of QIAamp® DNA Stool Mini Kit for ecological studies of gut microbiota. J. Microbiol. Methods 54:13–20.

Lilburn, Schmidt, and Breznak. 1999. Phylogenetic diversity of termite gut spirochaetes. Environ. Microbiol. 1:331–345.

Lim, J.Y., J. Yoon, and C.J. Hovde. 2010. A brief overview of Escherichia coli O157:H7 and its plasmid O157. J. Microbiol. Biotechnol. 20:5–14.

Liu, J., M. Zhang, R. Zhang, W. Zhu, and S. Mao. 2016. Comparative studies of the composition of bacterial microbiota associated with the ruminal content, ruminal epithelium and in the faeces of lactating dairy cows. Microb. Biotechnol. 9:257–68.

Low, J.C., I.J. McKendrick, C. McKechnie, D. Fenlon, S.W. Naylor, C. Currie, D.G.E. Smith, L. Allison, and D.L. Gaily. 2005. Rectal carriage of enterohemorrhagic Escherichia coli O157 in slaughtered cattle. Appl. Environ. Microbiol. 71:93–97.

Luby, C.., M.L.. Epp, M.. Jelinski, C.. Clark, and A.. Potter. 2017. A Comparison Of Treponema Groups In Cows With Irregular Heel Skin From Digital Dermatitis Endemic And A Digital Dermatitis Free Dairy. Page 45 in Proceedings of the 19th International Symposium and 11th Conference Lameness in Ruminants, Munich, Germany.

Luginbuhl, A., and M. Kollbrunner. 2000. Increasing number of cattle suffering from papillomatous digital dermatitis Switzerland. Schweiz. Arch. Tierheilkd. 142:513–519.

Mahalanabis, M., H. Al-Muayad, M.D. Kulinski, D. Altman, C.M. Klapperich, J. J. M. O'Brien, N.A. Ali, S.K. Aberegg, E. Abraham, G.R. Bernard, J.L. Vincent, P.F. Laterre, S.P. LaRosa, J.F. Dhainaut, A. Lopez-Rodriguez, J.S. Steingrub, G.E. Garber, J.D. Helterbrand, E.W. Ely, J. C. J. Fisher, K.E. Sands, D.W. Bates, P.N. Lanken, P.S. Graman, P.L. Hibberd, K.L. Kahn, J. Parsonnet, R. Panzer, E.J. Orav, D.R. Snydman, E. Black, J.S. Schwartz, R. Moore, J. B. L. Johnson, R. Platt, T. van der Poll, S.M. Opal, D.C. Angus, W.T. Linde-Zwirble, J. Lidicker, G. Clermont, J. Carcillo, M.R. Pinsky, D.C. Angus, R.S. Wax, D.G. Remick, S.P. Larosa, R.P. Peters, M.A. van Agtmael, S.A. Danner, P.H. Savelkoul, C.M. Vandenbroucke-Grauls, M.T. Cabeen, C. Jacobs-Wagner, W. Vollmer, J. V. Holtje, A.P. Middelberg, J. Geciova, D. Bury, P. Jelen, C. Lamanna, M.F. Mallette, O. Salazar, D. Di Carlo, K.H. Jeong, L.P. Lee, J. Wang, Z. Chen, P.L. Corstjens, M.G. Mauk, H.H. Bau, R.H. Liu, J. Yang, R. Lenigk, J. Bonanno, P. Grodzinski, A. Bhattacharyya, C.M. Klapperich, M.D. Kulinski, M. Mahalanabis, S. Gillers, J.Y. Zhang, S. Singh, C.M. Klapperich, A. Bhattacharyya, C.M. Klapperich, A. Bhattacharyya, C.M. Klapperich, R. Boom, C.J. Sol, M.M. Salimans, C.L. Jansen, P.M.W. Dillen, J. van der Noordaa, C. Lee, J. Kim, S.G. Shin, S. Hwang, A. Mahmoudpour, S. Rahimi, M. Sina, M.H. Soroush, S. Shahi, N. Asl-Aminabadi, N.H. Mendelson, J.J. Thwaites, J.J. Thwaites, U.C. Surana, J.M. Pfeffer, H. Strating, J.T. Weadge, A.J. Clarke, L.H. Chen, Q.J. Duan, M.T. Cai, Y.D. Wu, S.Q. Shang, S. Shang, G. Chen, Y. Wu, L. Du, Z. Zhao, J.A. Jordan, M.B. Durso, L.E. Lehmann, K.P. Hunfeld, T. Emrich, G. Haberhausen, H. Wissing, A. Hoeft, F. Stuber, A. Vince, S.Z. Lepej, B. Barsic, D. Dusek, Z. Mitrovic, R. Serventi-Seiwerth, B. Labar, A.S. Rossney,

C.M. Herra, G.I. Brennan, P.M. Morgan, B. O'Connell, and E.M. Marlowe. 2009. Cell lysis and DNA extraction of gram-positive and gram-negative bacteria from whole blood in a disposable microfluidic chip. Lab Chip 9:2811.

Maiden, M.C., J.A. Bygraves, E. Feil, G. Morelli, J.E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D.A. Caugant, I.M. Feavers, M. Achtman, and B.G. Spratt. 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc. Natl. Acad. Sci. U. S. A. 95:3140–5.

Manske, T., J. Hultgren, and C. Bergsten. 2002a. Topical treatment of digital dermatitis associated with severe heel-horn erosion in a Swedish dairy herd. Prev. Vet. Med. 53:215–231.

Manske, T., J. Hultgren, and C. Bergsten. 2002b. Prevalence and interrelationships of hoof lesions and lameness in Swedish dairy cows. Prev. Vet. Med. 54:247–263.

March, M.D., M.J. Haskell, M.G.G. Chagunda, F.M. Langford, and D.J. Roberts. 2014. Current trends in British dairy management regimens. J. Dairy Sci. 97:7985–94.

Mattman, L.H. 2001. Spirochete. In: Cell Wall Deficient Forms: Stealth Pathogens. 3rd Ed. Springer, Berlin.

Maukonen, J., C. Simões, and M. Saarela. 2012. The currently used commercial DNA-extraction methods give different results of clostridial and actinobacterial populations derived from human fecal samples. FEMS Microbiol. Ecol. 79:697–708.

McDonald, J.E., N. Larsen, A. Pennington, J. Connolly, C. Wallis, D.J. Rooks, N. Hall, A.J. McCarthy, and H.E. Allison. 2016. Characterising the Canine Oral Microbiome by Direct Sequencing of Reverse-Transcribed rRNA Molecules. PLoS One 11:e0157046.

Meyer, W., and K. Neurand. 1991. Comparison of skin pH in domesticated and laboratory mammals. Arch. Dermatol. Res. 283:16–18.

Miguel-Pacheco, G.G., J. Kaler, J. Remnant, L. Cheyne, C. Abbott, A.P. French, T.P. Pridmore, and J.N. Huxley. 2014. Behavioural changes in dairy cows with lameness in an automatic milking system. Appl. Anim. Behav. Sci. 150:1–8.

Milne, I., D. Lindner, M. Bayer, D. Husmeier, G. McGuire, D.F. Marshall, and F. Wright. 2009. TOPALi v2: a rich graphical interface for evolutionary analyses of multiple alignments on HPC clusters and multi-core desktops. Bioinformatics 25:126–7.

Miyamoto, M., K. Ishihara, and K. Okuda. 2006. The Treponema denticola surface protease dentilisin degrades interleukin-1 beta (IL-1 beta), IL-6, and tumor necrosis factor alpha. Infect. Immun. 74:2462–7.

Monteiro, L., D. Bonnemaison, A. Vekris, K.G. Petry, J. Bonnet, R. Vidal, J. Cabrita, and F. Mégraud. 1997. Complex polysaccharides as PCR inhibitors in feces: Helicobacter pylori model. J. Clin. Microbiol. 35:995–8.

Montenegro, M.A., M. Bülte, T. Trumpf, S. Aleksić, G. Reuter, E. Bulling, and R. Helmuth. 1990. Detection and characterization of fecal verotoxin-producing Escherichia coli from healthy cattle. J. Clin. Microbiol. 28:1417–21.

Moore, L.J., M.J. Woodward, and R. Grogono-Thomas. 2005. The occurrence of treponemes in contagious ovine digital dermatitis and the characterisation of associated

Dichelobacter nodosus. Vet. Microbiol. 111:199–209.

Moter, A., G. Leist, R. Rudolph, K. Schrank, B.K. Choi, M. Wagner, and U.B. Göbel. 1998. Fluorescence in situ hybridization shows spatial distribution of as yet uncultured treponemes in biopsies from digital dermatitis lesions. Microbiology 144:2459–2467.

Murgia, R., and M. Cinco. 2004. Induction of cystic forms by different stress conditions in Borrelia burgdorferi. APMIS 112:57–62.

Murray, R.., D.. Downham, I. Demirkan, and S.. Carter. 2002. Some relationships between spirochaete infections and digital dermatitis in four UK dairy herds. Res. Vet. Sci. 73:223–230.

Murray, R.D., D.Y. Downham, M.J. Clarkson, W.B. Faull, J.W. Hughes, F.J. Manson, J.B. Merritt, W.B. Russell, J.E. Sutherst, and W.R. Ward. 1996. Epidemiology of lameness in dairy cattle: description and analysis of foot lesions. Vet. Rec. 138:586–91.

Muzafar, M., L.E. Green, L.A. Calvo-Bado, E. Tichauer, H. King, P. James, and E.M.H. Wellington. 2016. Survival of the ovine footrot pathogen Dichelobacter nodosus in different soils. Anaerobe 38:81–7.

Nakamura, Y., M. Watanabe, W. Kamitani, H. Taniyama, T. Nakaya, Y. Nishimura, H. Tsujimoto, S. Machida, and K. Ikuta. 1999. High prevalence of Borna disease virus in domestic cats with neurological disorders in Japan. Vet. Microbiol. 70:153–169.

Nascimento, L.V., M.T. Mauerwerk, C.L. Dos Santos, I.R. de Barros Filho, E.H. Birgel Júnior, C.S. Sotomaior, H.M.F. Madeira, and R.D. Ollhoff. 2015. Treponemes detected in digital dermatitis lesions in brazilian dairy cattle and possible host reservoirs of infection. J. Clin. Microbiol. 53:1935–7.

Navarro, G., L.E. Green, and N. Tadich. 2013. Effect of lameness and lesion specific causes of lameness on time budgets of dairy cows at pasture and when housed. Vet. J. 197:788–93.

Naylor, S.W., J.C. Low, T.E. Besser, A. Mahajan, G.J. Gunn, M.C. Pearce, I.J. McKendrick, D.G.E. Smith, and D.L. Gally. 2003. Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic Escherichia coli O157:H7 in the bovine host. Infect. Immun. 71:1505–12.

Nelson-Filho, P., R.M.A. Valdez, M.C.D. Andrucioli, M.C.P. Saraiva, M. Feres, C.A. Sorgi, and L.H. Faccioli. 2011. Gram-negative periodontal pathogens and bacterial endotoxin in metallic orthodontic brackets with or without an antimicrobial agent: An in-vivo study. Am. J. Orthod. Dentofac. Orthop. 140:e281–e287.

Newbrook, K., G.J. Staton, S.R. Clegg, R.J. Birtles, S.D. Carter, and N.J. Evans. 2017. Treponema ruminis sp. nov., a spirochaete isolated from the bovine rumen. Int. J. Syst. Evol. Microbiol. 67:1349–1354.

Ng-Nguyen, D., S.F. Hii, V.-A.T. Nguyen, T. Van Nguyen, D. Van Nguyen, and R.J. Traub. 2015. Re-evaluation of the species of hookworms infecting dogs in Central Vietnam. Parasit. Vectors 8:401.

Nielsen, M.W., M.L. Strube, A. Isbrand, W.D.H.M. Al-Medrasi, M. Boye, T.K. Jensen, and K. Klitgaard. 2016. Potential bacterial core species associated with digital dermatitis in cattle herds identified by molecular profiling of interdigital skin samples. Vet.

Microbiol. 186:139–49.

Noordhoek, G.T., A. Cockayne, L.M. Schouls, R.H. Meloen, E. Stolz, and J.D. van Embden. 1990. A new attempt to distinguish serologically the subspecies of Treponema pallidum causing syphilis and yaws. J. Clin. Microbiol. 28:1600–1607.

Nordhoff, M. 2006. Biodiversity of bovine Treponema spp. Freie Universitat Berlin,.

Nordhoff, M., A. Moter, K. Schrank, and L.H. Wieler. 2008. High prevalence of treponemes in bovine digital dermatitis-a molecular epidemiology. Vet. Microbiol. 131:293–300.

Notomi, T., H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino, and T. Hase. 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28:63e–63.

Ohta, K., K.K. Makinen, and W.J. Loesche. 1986. Purification and characterization of an enzyme produced by Treponema denticola capable of hydrolyzing synthetic trypsin substrates. Infect. Immun. 53:213–20.

Oliveira, V.H.S., J.T. Sørensen, P.T. Thomsen, V. Machado, M. Gomez, D. Döpfer, C. Guard, R. Bicalho, N.J. Evans, C.A. Hart, and S.D. Carter. 2017. Associations between biosecurity practices and bovine digital dermatitis in Danish dairy herds. J. Dairy Sci. 10:e0120504.

Onyiro, O.M., L.J. Andrews, and S. Brotherstone. 2008. Genetic parameters for digital dermatitis and correlations with locomotion, production, fertility traits, and longevity in Holstein-Friesian dairy cows. J. Dairy Sci. 91:4037–4046.

Orsel, K., P. Plummer, J. Shearer, J. De Buck, S.D. Carter, R. Guatteo, and H.W. Barkema. 2017. Missing pieces of the puzzle to effectively control digital dermatitis. Transbound. Emerg. Dis.

Ouweltjes, W., M. Holzhauer, P.P.J. van der Tol, and J. van der Werf. 2009. Effects of two trimming methods of dairy cattle on concrete or rubber-covered slatted floors. J. Dairy Sci. 92:960–971.

Palmer, M.A., R.F. Donnelly, M.J. Garland, R. Majithiya, and N.E. O'Connell. 2013. The effect of slurry on skin permeability to methylene blue dye in dairy cows with and without a history of digital dermatitis. animal 7:1731–1737.

Palmer, M., and N. O'Connell. 2015. Digital Dermatitis in Dairy Cows: A Review of Risk Factors and Potential Sources of Between-Animal Variation in Susceptibility. Animals 5:512–535.

Parker, J., and M. Walker. 2011. Survival of a pathogenic Leptospira serovar in response to combined in vitro pH and temperature stresses. Vet. Microbiol. 152:146–50.

Paster, B.J., and E. Canale-Parola. 1985. Treponema saccharophilum sp. nov., a large pectinolytic spirochete from the bovine rumen. Appl. Environ. Microbiol. 50:212–219.

Pearce, M.C., D. Fenlon, J.C. Low, A.W. Smith, H.I. Knight, J. Evans, G. Foster, B.A. Synge, and G.J. Gunn. 2004. Distribution of Escherichia coli O157 in bovine fecal pats and its impact on estimates of the prevalence of fecal shedding. Appl. Environ. Microbiol. 70:5737–43.

Petri, R.M., T. Schwaiger, G.B. Penner, K.A. Beauchemin, R.J. Forster, J.J. McKinnon, and

T.A. McAllister. 2013. Characterization of the core rumen microbiome in cattle during transition from forage to concentrate as well as during and after an acidotic challenge. PLoS One 8:e83424.

Phillips, N.D., T. La, and D.J. Hampson. 2010. Survival of intestinal spirochaete strains from chickens in the presence of disinfectants and in faeces held at different temperatures. Avian Pathol.

Picard, C., C. Ponsonnet, E. Paget, X. Nesme, and P. Simonet. 1992. Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. Appl. Environ. Microbiol. 58:2717–22.

Pitta, D.W., W.E. Pinchak, S.E. Dowd, J. Osterstock, V. Gontcharova, E. Youn, K. Dorton, I. Yoon, B.R. Min, J.D. Fulford, T.A. Wickersham, and D.P. Malinowski. 2010. Rumen Bacterial Diversity Dynamics Associated with Changing from Bermudagrass Hay to Grazed Winter Wheat Diets. Microb. Ecol. 59:511–522.

Pond, S.L.K., and S.D.W. Frost. 2005. Datamonkey: rapid detection of selective pressure on individual sites of codon alignments. Bioinformatics 21:2531–2533.

Pontiroli, A., E.R. Travis, F.P. Sweeney, D. Porter, W.H. Gaze, S. Mason, V. Hibberd, J. Holden, O. Courtenay, E.M.H. Wellington, J. Young, E. Gormley, E. Wellington, F. Sweeney, O. Courtenay, A. Ul-Hassan, V. Hibberd, L. Reilly, C. Donnelly, R. Woodroffe, D. Cox, J. Bourne, G. Gettinby, J. Coleman, M. Coleman, B. Warburton, P. Caley, G. Hickling, P. Cowan, D. Pfeiffer, S. Dokoupil, I. Genov, E. Maddock, R. Williams, W. Hoy, J. Young, E. Gormley, E. Wellington, O. Courtenay, L. Reilly, F. Sweeney, V. Hibberd, S. Bryan, O. Courtenay, E. Wellington, L. Reilly, O. Courtenay, A. Hiroshi, I. Akiko, A. Eiji, M. Sou-ichi, O. Yumiko, F. Sweeney, O. Courtenay, V. Hibberd, R. Hewinson, L. Reilly, P. Lantz, M. Matsson, T. Wadström, P. Rådström, J. Ghosh, P. Larsson, B. Singh, B. Pettersson, N. Islam, Y. Tsai, B. Olson, R. Griffiths, A. Whiteley, A. O'Donnell, M. Bailey, D. Dong, A. Yan, H. Liu, X. Zhang, Y. Xu, M. Sagova-Mareckova, L. Cermak, J. Novotna, K. Plhackova, J. Forstova, K. Purdy, T. Embley, S. Takii, D. Nedwell, S. Obika, D. Nanbu, Y. Hari, J. Andoh, O. Mori, A. Simeonov, T. Nikiforov, B. Holland, M. Copenhaver, R. Artz, L. Avery, D. Jones, K. Killham, M. Kaser, M. Ruf, J. Hauser, L. Marsollier, G. Pluschke, H. Chen, M. Rangasamy, S. Tan, H. Wang, B. Siegfried, W. Aldous, J. Pounder, J. Cloud, G. Woods, C. Whitehouse, H. Hottel, M. Queipo-Ortuño, F. Tena, J. Colmenero, P. Morata, L. Durnez, P. Stragier, K. Roebben, A. Ablordey, H. Leirs, M. Inoue, W. Tang, S. Wee, T. Barkham, L. Hartman, S. Coyne, D. Norwood, S. Dineen, R. Aranda, D. Anders, and J. Robertson. 2011. Pathogen Quantitation in Complex Matrices: A Multi-Operator Comparison of DNA Extraction Methods with a Novel Assessment of PCR Inhibition. PLoS One 6:e17916.

Porcella, S.F., and T.G. Schwan. 2001. Borrelia burgdorferi and Treponema pallidum: a comparison of functional genomics, environmental adaptations, and pathogenic mechanisms. J. Clin. Invest. 107:651–6.

Pringle, M., A. Backhans, F. Otman, M. Sjölund, and C. Fellström. 2009. Isolation of spirochetes of genus Treponema from pigs with ear necrosis. Vet. Microbiol. 139:279–283.

Radolf, J.D., R.K. Deka, A. Anand, D. Šmajs, M. V. Norgard, and X.F. Yang. 2016. Treponema pallidum, the syphilis spirochete: making a living as a stealth pathogen. Nat. Rev. Microbiol. 14:744–759.

Radolf, J.D., and S.A. Lukehart. 2006. Pathogenic Treponema : Molecular and Cellular Biology. Caister Academic Press, Norfolk, UK.

Rantakokko-Jalava, K., and J. Jalava. 2002. Optimal DNA isolation method for detection of bacteria in clinical specimens by broad-range PCR. J. Clin. Microbiol. 40:4211–7.

Rapp, D. 2010. DNA extraction from bovine faeces: current status and future trends. J. Appl. Microbiol. 108:1485–93.

Rasmussen, M., N. Capion, K. Klitgaard, T. Rogdo, T. Fjeldaas, M. Boye, and T.K. Jensen. 2012. Bovine digital dermatitis: possible pathogenic consortium consisting of Dichelobacter nodosus and multiple Treponema species. Vet. Microbiol. 160:151–61.

Read, D., and R. Walker. 1996. Experimental transmission of papillomatous digital dermatitis (footwarts) in cattle. Vet. Pathol. 33:607.

Read, D.H., and R.L. Walker. 1998. Papillomatous digital dermatitis (footwarts) in California dairy cattle: clinical and gross pathologic findings. J. Vet. Diagnostic Investig. 10:67–76.

Read, D.H., R.L. Walker, A.E. Castro, J.P. Sundberg, and M.C. Thurmond. 1992. An invasive spirochaete associated with interdigital papillomatosis of dairy cattle. Vet. Rec. 130:59–60.

Rebhun, W.C., R.M. Payne, J.M. King, M. Wolfe, and S.N. Begg. 1980. Interdigital papillomatosis in dairy cattle. J. Am. Vet. Med. Assoc. 177:437–440.

Relun, A., R. Guatteo, M.M. Auzanneau, and N. Bareille. 2013a. Farmers' practices, motivators and barriers for adoption of treatments of digital dermatitis in dairy farms. Animal 7:1542–50.

Relun, A., R. Guatteo, P. Roussel, and N. Bareille. 2011. A simple method to score digital dermatitis in dairy cows in the milking parlor. J. Dairy Sci. 94:5424–5434.

Relun, A., A. Lehebel, M. Bruggink, N. Bareille, and R. Guatteo. 2013b. Estimation of the relative impact of treatment and herd management practices on prevention of digital dermatitis in French dairy herds. Prev. Vet. Med. 110:558–62.

Robbins, R.C., V.C. Artuso-Ponte, A.J. Moeser, W.E. Morgan Morrow, J.W. Spears, and W.A. Gebreyes. 2013. Effects of quaternary benzo(c)phenanthridine alkaloids on growth performance, shedding of organisms, and gastrointestinal tract integrity in pigs inoculated with multidrug-resistant Salmonella spp. Am. J. Vet. Res. 74:1530–1535.

Robinson, S.E., P.E. Brown, E. John Wright, M. Bennett, C.A. Hart, and N.P. French. 2005. Heterogeneous distributions of Escherichia coli O157 within naturally infected bovine faecal pats. FEMS Microbiol. Lett. 244.

Rodríguez-Lainz, A., D.W. Hird, T.E. Carpenter, and D.H. Read. 1996. Case-control study of papillomatous digital dermatitis in Southern California dairy farms. Prev. Vet. Med. 28:117–131.

Rodriguez-Lainz, A., P. Melendez-Retamal, D.W. Hird, and D.H. Read. 1998. Papillomatous digital dermatitis in Chilean dairies and evaluation of a screening method. Prev. Vet. Med. 37:197–207.

Rodriguez-Lainz, A., P. Melendez-Retamal, D.W. Hird, D.H. Read, and R.L. Walker. 1999. Farm- and host-level risk factors for papillomatous digital dermatitis in Chilean dairy cattle. Prev. Vet. Med. 42:87–97.

Rosen, G., T. Genzler, and M.N. Sela. 2008. Coaggregation of Treponema denticola with Porphyromonas gingivalis and Fusobacterium nucleatum is mediated by the major outer sheath protein of Treponema denticola. FEMS Microbiol. Lett. 289:59–66.

Rougemont, M., M. Van Saanen, R. Sahli, H.P. Hinrikson, J. Bille, and K. Jaton. 2004. Detection of Four Plasmodium Species in Blood from Humans by 18S rRNA Gene Subunit-Based and Species-Specific Real-Time PCR Assays. J. Clin. Microbiol. 42:5636–5643.

Rudi, K., H.K. Høidal, T. Katla, B.K. Johansen, J. Nordal, and K.S. Jakobsen. 2004. Direct real-time PCR quantification of Campylobacter jejuni in chicken fecal and cecal samples by integrated cell concentration and DNA purification. Appl. Environ. Microbiol. 70:790–7.

Rurangirwa, F.R., P.M. Dilbeck, T.B. Crawford, T.C. McGuire, and T.F. McElwain. 1999. Analysis of the 16S rRNA gene of micro-organism WSU 86-1044 from an aborted bovine foetus reveals that it is a member of the order Chlamydiales: proposal of Waddliaceae fam. nov., Waddlia chondrophila gen. nov., sp. nov.. Int. J. Syst. Bacteriol. 49 Pt 2:577–81.

Salonen, A., J. Nikkilä, J. Jalanka-Tuovinen, O. Immonen, M. Rajilić-Stojanović, R.A. Kekkonen, A. Palva, and W.M. de Vos. 2010. Comparative analysis of fecal DNA extraction methods with phylogenetic microarray: effective recovery of bacterial and archaeal DNA using mechanical cell lysis. J. Microbiol. Methods 81:127–34.

Salthammer, T. 2015. The formaldehyde dilemma. Int. J. Hyg. Environ. Health 218:433–436.

Santos, T.M.A., R. V. Pereira, L.S. Caixeta, C.L. Guard, and R.C. Bicalho. 2012. Microbial diversity in bovine papillomatous digital dermatitis in Holstein dairy cows from upstate New York. FEMS Microbiol. Ecol. 79:518–529.

Sayers, G., P.X. Marques, N.J. Evans, L. O'Grady, M.L. Doherty, S.D. Carter, and J.E. Nally. 2009. Identification of spirochetes associated with contagious ovine digital dermatitis. J. Clin. Microbiol. 47:1199–201.

Schlafer, S., M. Nordhoff, C. Wyss, S. Strub, J. Hübner, D.M. Gescher, A. Petrich, U.B. Göbel, and A. Moter. 2008. Involvement of Guggenheimella bovis in digital dermatitis lesions of dairy cows. Vet. Microbiol. 128:118–25.

Schleifer, K.H., and O. Kandler. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. 36:407–77.

Schöpke, K., S. Weidling, R. Pijl, and H.H. Swalve. 2013. Relationships between bovine hoof disorders, body condition traits, and test-day yields. J. Dairy Sci. 96:679–689.

Scola, B. La, L.T.M. Bui, G. Baranton, A. Khamis, D. Raoult, V. Morel, M. Reis, A. Ko, O. Dellagostin, and D. Alt. 2006. Partial rpoB gene sequencing for identification of Leptospira species. FEMS Microbiol. Lett. 263:142–147.

Shearer, J., P. Plummer, and A. Krull. 2016. Digital Dermatitis: New Ideas on an Old Disease. Pages 54–61 in Proceedings of the first regional conference on cow comfort and

lameness (RCCCL), Tehran, Iran.

Shibahara, T., T. Ohya, R. Ishii, Y. Ogihara, T. Maeda, Y. Ishikawa, and K. Kadota. 2002. Concurrent spirochaetal infections of the feet and colon of cattle in Japan. Aust Vet J 80:497–502.

Singh, A.E., and B. Romanowski. 1999. Syphilis: review with emphasis on clinical, epidemiologic, and some biologic features. Clin. Microbiol. Rev. 12:187–209.

Singhal, N., M. Kumar, P.K. Kanaujia, and J.S. Virdi. 2015. MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. Front. Microbiol. 6:791.

Solano, L., H.W.H. Barkema, C. Jacobs, and K. Orsel. 2017. Validation of the M-stage scoring system for digital dermatitis on dairy cows in the milking parlor. J. Dairy Sci. 100:1592–1603.

Somers, J.G.C.J., K. Frankena, E.N. Noordhuizen-Stassen, and J.H.M. Metz. 2003. Prevalence of claw disorders in Dutch dairy cows exposed to several floor systems. J. Dairy Sci. 86:2082–93.

Somers, J.G.C.J., K. Frankena, E.N. Noordhuizen-Stassen, and J.H.M. Metz. 2005. Risk factors for digital dermatitis in dairy cows kept in cubicle houses in The Netherlands. Prev. Vet. Med. 71:11–21.

Songer, J.G., and D.L. Harris. 1978. Transmission of swine dysentery by carrier pigs. Am. J. Vet. Res. 39:913–916.

de Souza, L., and M.C. Koury. 1992. Isolation and biological activities of endotoxin from Leptospira interrogans. Can. J. Microbiol. 38:284–9.

Speijers, M.H.M., L.G. Baird, G.A. Finney, J. McBride, D.J. Kilpatrick, D.N. Logue, and N.E. O'Connell. 2010. Effectiveness of different footbath solutions in the treatment of digital dermatitis in dairy cows. J. Dairy Sci. 93:5782–5791.

Stamm, L. V, H.L. Bergen, and R.L. Walker. 2002. Molecular Typing of Papillomatous Digital Dermatitis-Associated Treponema Isolates Based on Analysis of 16S-23S Ribosomal DNA Intergenic Spacer Regions. J. Clin. Microbiol. 40:3463–3469.

Stanton, T.B., and E. Canale-Parola. 1980. Treponema bryantii sp. nov., a rumen spirochete that interacts with cellulolytic bacteria. Arch. Microbiol. 127:145–156.

Staton, G.J., K. Newbrook, S.R. Clegg, R.J. Birtles, N.J. Evans, and S.D. Carter. 2017. Treponema rectale sp. nov., a spirochete isolated from the bovine rectum. Int. J. Syst. Evol. Microbiol.

Stokes, J.E. 2011. Investigating novel and existing methods of preventing, detecting and treating digital dermatitis in dairy cows. University of Bristol,.

Stokes, J.E., K.A. Leach, D.C.J. Main, and H.R. Whay. 2012a. The reliability of detecting digital dermatitis in the milking parlour. Vet. J. 193:679–684.

Stokes, J.E., K.A. Leach, D.C.J. Main, and H.R. Whay. 2012b. An investigation into the use of infrared thermography (IRT) as a rapid diagnostic tool for foot lesions in dairy cattle. Vet. J. 193:674–678.

Strub, S., J.R. Van Der Ploeg, K. Nuss, C. Wyss, A. Luginbühl, A. Steiner, A. Luginbhl, and A. Steiner. 2007. Quantitation of Guggenheimella bovis and treponemes in bovine tissues related to digital dermatitis. FEMS Microbiol. Lett. 269:48–53.

Sullivan, L.E. 2015. Identifying Digital Dermatitis infection reservoirs in beef cattle and sheep. University of Liverpool,.

Sullivan, L.E., R.W. Blowey, S.D. Carter, J.S. Duncan, D.H. Grove-White, P. Page, T. Iveson, J.W. Angell, and N.J. Evans. 2014a. Presence of digital dermatitis treponemes on cattle and sheep hoof trimming equipment. Vet. Rec. 175:201.

Sullivan, L.E., S.D. Carter, R. Blowey, J.S. Duncan, D. Grove-White, and N.J. Evans. 2013. Digital dermatitis in beef cattle. Vet. Rec. 173:582.

Sullivan, L.E., S.D. Carter, J.S. Duncan, D.H. Grove-White, J.W. Angell, and N.J. Evans. 2015a. The gastrointestinal tract as a potential infection reservoir of digital dermatitis treponemes in beef cattle and sheep. Appl. Environ. Microbiol. AEM.01956-15-.

Sullivan, L.E., S.R. Clegg, J.W. Angell, K. Newbrook, R.W. Blowey, S.D. Carter, J. Bell, J.S. Duncan, D.H. Grove-White, R.D. Murray, and N.J. Evans. 2015b. The high association of bovine digital dermatitis Treponema spp. with contagious ovine digital dermatitis lesions and the presence of Fusobacterium necrophorum and Dichelobacter nodosus. J. Clin. Microbiol. JCM.00180-15.

Sullivan, L.E., N.J. Evans, R.W. Blowey, D.H. Grove-White, S.R. Clegg, J.S. Duncan, and S.D. Carter. 2015c. A molecular epidemiology of treponemes in beef cattle digital dermatitis lesions and comparative analyses with sheep contagious ovine digital dermatitis and dairy cattle digital dermatitis lesions. Vet. Microbiol.

Sullivan, L.E., N.J. Evans, S.R. Clegg, S.D. Carter, J.E. Horsfield, D. Grove-White, and J.S. Duncan. 2014b. Digital dermatitis treponemes associated with a severe foot disease in dairy goats. Vet. Rec. vetrec-2014-102858.

Suthar, V.S., O. Burfeind, J.S. Patel, A.J. Dhami, W. Heuwieser, W. Heuwieser, and R.D. Randel. 2011. Body temperature around induced estrus in dairy cows. J. Dairy Sci. 94:2368–73.

Svartström, O., F. Karlsson, C. Fellström, and M. Pringle. 2013. Characterization of Treponema spp. isolates from pigs with ear necrosis and shoulder ulcers. Vet. Microbiol. 166:617–623.

Sykora, S., K. Pieber, H. Simhofer, V. Hackl, D. Brodesser, and S. Brandt. 2014. Isolation of Treponema and Tannerella spp. from equine odontoclastic tooth resorption and hypercementosis related periodontal disease. Equine Vet. J. 46:358–363.

Szafrański, S.P., A. Winkel, and M. Stiesch. 2017. The use of bacteriophages to biocontrol oral biofilms. J. Biotechnol. 250:29–44.

Tamura, K., and M. Nei. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10:512–26.

Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30:2725–9.

Tang, J., Z. Zeng, H. Wang, T. Yang, P. Zhang, Y. Li, A. Zhang, W. Fan, Y. Zhang, X. Yang, S. Zhao, G. Tian, and L. Zou. 2008. An effective method for isolation of DNA from pig faeces and comparison of five different methods. J. Microbiol. Methods 75:432–6.

Telezhenko, E., L. Lidfors, and C. Bergsten. 2007. Dairy Cow Preferences for Soft or Hard Flooring when Standing or Walking. J. Dairy Sci. 90:3716–3724.

Thomsen, P.T., A.K. Ersbøll, and J.T. Sørensen. 2012. Short communication: Automatic washing of hooves can help control digital dermatitis in dairy cows. J. Dairy Sci. 95:7195–9.

Thomsen, P.T., I.C. Klaas, and K. Bach. 2008. Short Communication: Scoring of Digital Dermatitis During Milking as an Alternative to Scoring in a Hoof Trimming Chute. J. Dairy Sci. 91:4679–4682.

Timms, L. 2008. Preliminary Evaluation of Separated Manure Solids Characteristics at the New ISU Dairy. Anim. Ind. Rep. 654.

Trochimchuk, T., J. Fotheringham, E. Topp, H. Schraft, and K.T. Leung. 2003. A comparison of DNA extraction and purification methods to detect Escherichia coli O157:H7 in cattle manure. J. Microbiol. Methods 54:165–175.

Trott, D.J., M.R. Moeller, R.L. Zuerner, J.P. Goff, W.R. Waters, D.P. Alt, R.L. Walker, and M.J. Wannemuehler. 2003. Characterization of Treponema phagedenis-Like Spirochetes Isolated from Papillomatous Digital Dermatitis Lesions in Dairy Cattle. J. Clin. Microbiol. 41:2522–2529.

Vermunt, J., and F. Hill. 2004. Papillomatous digital dermatitis in a Holstein-Friesian bull. N. Z. Vet. J. 52:99–101.

Versteeg, B., M.S. van Rooijen, M.F. Schim van der Loeff, H.J.C. de Vries, and S.M. Bruisten. 2014. No indication for tissue tropism in urogenital and anorectal Chlamydia trachomatis infections using high-resolution multilocus sequence typing. BMC iInfectious Dis. 14:464.

Vink, W.D. 2006. Investigating the epidemiology of Bovine Digital Dermatitis: causality, transmission and infection dynamics. University of Liverpool,.

Vokey, F.J., C.L. Guard, H.N. Erb, and D.M. Galton. 2001. Effects of alley and stall surfaces on indices of claw and leg health in dairy cattle housed in a free-stall barn. J. Dairy Sci. 84:2686–2699.

Walker, R.L., D.H. Read, K.J. Loretz, and R.W. Nordhausen. 1995. Spirochetes isolated from dairy cattle with papillomatous digital dermatitis and interdigital dermatitis. Vet. Microbiol. 47:343–55.

Ward, W.R., J.W. Hughes, W.B. Faull, P.J. Cripps, J.P. Sutherland, and J.E. Sutherst. 2002. Observational study of temperature, moisture, pH and bacteria in straw bedding, and faecal consistency, cleanliness and mastitis in cows in four dairy herds. Vet. Rec. 151:199–206.

Warnick, L.D., D. Janssen, C.L. Guard, and Y.T. Grohn. 2001. The Effect of Lameness on Milk Production in Dairy Cows. J. Dairy Sci. 84:1988–1997.

Watters, M.E.A., K.M.A. Meijer, H.W. Barkema, K.E. Leslie, M.A.G. von Keyserlingk, and T.J.

Devries. 2013. Associations of herd- and cow-level factors, cow lying behavior, and risk of elevated somatic cell count in free-stall housed lactating dairy cows. Prev. Vet. Med. 111:245–55.

Weingarden, A.R., C. Chen, A. Bobr, D. Yao, Y. Lu, V.M. Nelson, M.J. Sadowsky, and A. Khoruts. 2014. Microbiota transplantation restores normal fecal bile acid composition in recurrent Clostridium difficile infection. AJP Gastrointest. Liver Physiol. 306:G310–G319.

Weiss, S., A. Menezes, K. Woods, A. Chanthongthip, S. Dittrich, A. Opoku-Boateng, M. Kimuli, and V. Chalker. 2016. An Extended Multilocus Sequence Typing (MLST) Scheme for Rapid Direct Typing of Leptospira from Clinical Samples. PLoS Negl. Trop. Dis. 10:e0004996.

Wells, S.., L.. Garber, and B.. Wagner. 1999. Papillomatous digital dermatitis and associated risk factors in US dairy herds. Prev. Vet. Med. 38:11–24.

Westphal, A., M.L. Williams, F. Baysal-Gure, J.T. LeJeune, and B.B.M.S. Gardener. 2011. General suppression of Escherichia coli O157:H7 in sand-based dairy livestock bedding. Appl. Environ. Microbiol. 77:2113–2121.

WHO. 2011. Critically Important Antimicrobials for Human Medicine. 3rd Revisi. World Health Organization, Geneva, Switzerland.

Willshire, J.A., and N.J. Bell. 2009. An economic review of cattle lameness. Pages 136–141 in Cattle Practice.

Wilson-Welder, J.H., D.P. Alt, and J.E. Nally. 2015. Digital Dermatitis in Cattle: Current Bacterial and Immunological Findings. Anim. an open access J. from MDPI 5:1114–1135.

Wilson-Welder, J.H., M.K. Elliott, R.L. Zuerner, D.O. Bayles, D.P. Alt, and T.B. Stanton. 2013. Biochemical and molecular characterization of Treponema phagedenis-like spirochetes isolated from a bovine digital dermatitis lesion. BMC Microbiol. 13:280.

Wilson, I.G. 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. 63:3741–51.

Wyss, C. 1992. Growth of Porphyromonas gingivalis, Treponema denticola, T. pectinovorum, T. socranskii, and T. vincentii in a chemically defined medium. J. Clin. Microbiol. 30:2225–2229.

Wyss, C., F.E. Dewhirst, B.J. Paster, T. Thurnheer, and A. Luginbühl. 2005. Guggenheimella bovis gen. nov., sp. nov., isolated from lesions of bovine dermatitis digitalis. Int. J. Syst. Evol. Microbiol. 55:667–671.

Yano, T., K.K. Moe, T. Chuma, N. Misawa, M. NEI, S. KUMAR, J.S. DUNCAN, S. EFSTATHIOU, R.J. BOWDEN, C. CHASTEL, M. BENNETT, J.T. SAMPLE, B. BARRELL, A.J. DAVISON, and J.P. STEWART. 2010a. Antimicrobial susceptibility of Treponema phagedenis-like spirochetes isolated from dairy cattle with papillomatous digital dermatitis lesions in Japan. J. Vet. Med. Sci. 72:379–82.

Yano, T., K.K. Moe, K. Yamazaki, T. Ooka, T. Hayashi, and N. Misawa. 2010b. Identification of candidate pathogens of papillomatous digital dermatitis in dairy cattle from quantitative 16S rRNA clonal analysis. Vet. Microbiol. 143:352–362.

Yano, T., R. Yamagami, K. Misumi, C. Kubota, K.K. Moe, T. Hayashi, K. Yoshitani, O. Ohtake, and N. Misawa. 2009. Genetic heterogeneity among strains of Treponema phagedenis-like spirochetes isolated from dairy cattle with papillomatous digital dermatitis in Japan. J. Clin. Microbiol. 47:727–33.

Yao, E.S., R.J. Lamont, S.P. Leu, and A. Weinberg. 1996. Interbacterial binding among strains of pathogenic and commensal oral bacterial species. Oral Microbiol. Immunol. 11:35–41.

Yarnell, K., M. Le Bon, N. Turton, M. Savova, A. McGlennon, and S. Forsythe. 2017. Reducing exposure to pathogens in the horse: a preliminary study into the survival of bacteria on a range of equine bedding types. J. Appl. Microbiol. 122:23–29.

Yazdankhah, S.P., P. Kriz, G. Tzanakaki, J. Kremastinou, J. Kalmusova, M. Musilek, T. Alvestad, K.A. Jolley, D.J. Wilson, N.D. McCarthy, D.A. Caugant, and M.C.J. Maiden. 2004. Distribution of serogroups and genotypes among disease-associated and carried isolates of Neisseria meningitidis from the Czech Republic, Greece, and Norway. J. Clin. Microbiol. 42:5146–5153.

You, M., S. Mo, W.K. Leung, R.M. Watt, M. Matsumoto-Nakano, F. Asai, T. Ooshima, and H. Hazewinkel. 2013. Comparative analysis of oral treponemes associated with periodontal health and disease. BMC Infect. Dis. 13:174.

Zdanowicz, M., J.A. Shelford, C.B. Tucker, D.M. Weary, M.A.G. von Keyserlingk, W.P. Weiss, K.L. Smith, G.L. Bowman, L.E. Heider, B.L. Brockett, and H.R. Conrad. 2004. Bacterial populations on teat ends of dairy cows housed in free stalls and bedded with either sand or sawdust. J. Dairy Sci. 87:1694–701.

Zinicola, M., H. Higgins, S. Lima, V. Machado, C. Guard, and R. Bicalho. 2015a. Shotgun Metagenomic Sequencing Reveals Functional Genes and Microbiome Associated with Bovine Digital Dermatitis. PLoS One 10:e0133674.

Zinicola, M., F. Lima, S. Lima, V. Machado, M. Gomez, D. Döpfer, C. Guard, and R. Bicalho. 2015b. Altered microbiomes in bovine digital dermatitis lesions, and the gut as a pathogen reservoir. PLoS One 10:e0120504.

Appendix A

Supplementary material relating to Chapter 3 and Chapter 4

On the next few pages are the full manufacturer's protocols for each of the commercial DNA extraction kits described in Chapter 3 and 4 including any modifications to the protocols made by the author.

DNeasy® Blood and Tissue Kit (QT; Qiagen, Manchester, UK)

Tissue samples and swabs were cut into smaller pieces using sterile scalpel blades and ≤ 25 mg of each sample were placed into 1.5 ml microcentrifuge tubes. For DD treponeme spiked faecal and culture samples (DNA extraction method QT in Chapter 3), 20 mg was measured into a 1.5 ml microcentrifuge tube per sample. To each starting amount of sample, 180 µl of ATL Buffer and 20 µl Proteinase K were added and vortexed to mix before incubating at 56°C overnight in a rocking incubator until the samples were fully lysed.

Following lysis the samples were vortexed immediately for 15 seconds to mix and 200 µl of Buffer AL was added and mixed again by vortexing. Then 200 µl of 100% molecular grade ethanol (Sigma-Aldrich, Dorset, UK) was added and vortexed to mix.

The lysed sample mixtures were then transferred into DNeasy Mini Spin Columns that were placed in 2 ml collection tubes provided with the kit. Spin columns were then centrifuged at 6000 *g* for 1 minute and the collection tubes with the filtrate were discarded.

The spin columns were placed in new 2 ml collection tubes and washed by adding 500 µl Buffer AW1 to the top of the spin columns and centrifuged at 6000 *g* for 1 minute. The collection tubes containing the filtrate were discarded.

The spin columns were placed in new 2 ml collection tubes and washed a second time by adding 500 µl of Buffer AW2 and centrifuged at 20 000 *g* for 3 minutes. The collection tubes containing filtrate were discarded.

Following washing, the spin columns were then transferred to 1.5 ml microcentrifuge tubes and 100 µl of Buffer AE was added to the centre of the spin columns before incubating at ambient temperature for 1 minute. The spin columns were then centrifuged at 6000 *g* for 1 minute. The spin columns were discarded and the eluted genomic DNA in the 1.5 ml microcentrifuge tubes were stored at -20 °C.

QIA® Fast DNA Stool Mini Kit (QS; Qiagen, Manchester, UK)

An initial pretreatment step of adding 200 mg of DD treponeme spiked faecal samples to 1.4 ml ASL Buffer (provided in the QIAamp® DNA Stool Mini Kit, Qiagen, Manchester, UK) followed by incubating at 70°C for 10 minutes was carried out for DNA extraction method QS.2 as described by Klitgaard et al., (2014). The DNA extraction method then followed the manufacturer's protocol onwards from the addition of 1 ml InhibitEX Buffer described

below. DNA extraction methods QS and QS.1 in Chapter 3 followed the standard manufacturer's protocol from the beginning as described below with no initial pre-treatment step.

A starting amount of 200 mg for each DD treponeme spiked faecal sample and DD treponeme culture sample was measured and placed into a 2 ml microcentrifuge tube on ice and 1 ml of InhibitEX Buffer was added to each sample microcentrifuge tube. The samples containing the InhibitEX Buffer were then vortexed for 1 minute or until the mixtures were completely homogenised and then incubated at 70°C for 5 minutes, followed by vortexing for 15 seconds. Alternatively for DNA extraction method QS.1 in Chapter 3, the incubation temperature was increased to 95°C as suggested in the manufacturer's protocol for difficult to lyse bacteria.

Solid material within the samples (i.e. faecal particles) were then pelleted by centrifuging at 20 000 *g* for 1 minute and 200 µl of each sample supernatant was transferred into a 1.5 ml microcentrifuge tube containing 15 µl Proteinase K, to which 200 µl of Buffer AL was then added. Following vortexing for 15 seconds the sample supernatant mixtures were incubated at 70°C for 10 minutes. After incubation 200 µl of 100% molecular grade ethanol (Sigma-Aldrich, Dorset, UK) was added and vortexed briefly.

From the previous step, 600 µl of each sample lysate was transferred to QIAamp spin columns placed within 2 ml collection tubes and centrifuged at 20 000 *g* for 1 minute after which the 2 ml collection tubes containing filtrate were discarded.

The spin columns were then placed in new 2 ml collection tubes and washed by adding 500 µl Buffer AW1 to the top of the spin columns and centrifuging at 20 000 *g* for 1 minute. The collection tubes containing filtrate were discarded.

The spin columns were again placed in new 2 ml collection tubes and washed a second time by adding 500 µl of Buffer AW2 and centrifuged at 20 000 *g* for 3 minutes. The collection tubes containing filtrate were discarded. The spin columns placed in new 2 ml collection tubes were then centrifuged for a further 3 minutes to ensure any residual Buffer AW2 was removed and the collection tubes were discarded.

For the elution step, the spin columns were transferred to a 1.5 ml microcentrifuge tube and 200 µl of Buffer ATE was added to the centre of the spin columns before incubating at ambient temperature for 1 minute. Following incubation the spin columns were

centrifuged at 20 000 *g* for 1 minute and the eluted genomic DNA in the 1.5 ml microcentrifuge was stored at -20°C . The spin column was discarded.

Wizard® Genomic DNA Purification Kit (PW; Promega, Southampton, UK)

DD treponeme spiked faecal samples underwent an initial preparation step in which they were diluted ten-fold in 1X PBS and the faecal material was allowed to sediment for 2 minutes. The manufacturer's protocol was then followed using the liquid above the sedimented material. A starting amount of 1 ml of each pre-prepared DD treponeme spiked faecal sample or DD treponeme culture sample was transferred into individual 1.5 ml centrifuge tubes and cells were pelleted by centrifuging at 16 000 *g* for 2 minutes and the supernatant discarded.

Cell pellets were resuspended in 600 µl of Nuclei Lysis Solution by gentle pipetting and then incubated at 80°C for 5 minutes to enable cell lysis. Once cooled to room temperature, 3 µl of RNase Solution was added to the cell lysates and mixed by inverting five times before incubating at 37°C for 60 minutes. After cooling to room temperature, 200 µl of Protein Precipitation Solution was added to the cell lysates and mixed by vortexing at maximum speed for 20 seconds. The lysates were then incubated on ice for 5 minutes before centrifuging at 16 000 *g* at 4°C for 5 minutes (temperature and time are modifications of the manufacturer's protocol). The supernatants were then transferred to new 1.5 ml microcentrifuge tubes to which 600 µl ambient temperature isoproponal (Sigma-Aldrich, Dorset, UK) had already been added. As an addition to the manufacturer's protocol, the previous centrifugation step was repeated for 3 minutes with the original cell lysates and the supernatants were added to the 1.5 ml microcentrifuge tubes containing supernatant and isoproponal.

The supernatants were then mixed with the isoproponal by gently inverting the tubes until visible masses of thread-like strands of DNA were visible in each tube. The tubes containing the precipitated DNA were then centrifuged at 16 000 *g* at 4°C for 3 minutes (temperature and time are modifications of the manufacturer's protocol). The supernatants were then removed and the tubes allowed to drain on clean absorbent paper. The DNA pellet was then washed with 600 µl of ambient temperature 70% molecular grade ethanol (Sigma-Aldrich, Dorset, UK) by inverting the tubes gently several times and centrifuging at 16 000 *g* at 4°C for 3 minutes (temperature and time are modifications of the manufacturer's

protocol). The ethanol was then removed and the tubes allowed to drain on clean absorbent paper before the DNA pellet was air dried for 15 minutes. The DNA was then rehydrated by adding 100 µl of DNA Rehydration Solution and incubating at 65°C for 1 hour with periodic mixing by tapping the tubes. The rehydrated DNA was stored at -20°C.

Powersoil® DNA Isolation Kit (MP; MO BIO laboratories Inc, Carlsbad, CA, USA)

For DNA extraction methods MP and MP.2 (Chapter 3) a starting amount of 250 mg of the DD treponeme spiked faecal samples and DD treponeme culture samples were transferred into the PowerBead Tubes provided with the kit and mixed with the contents of the PowerBead Tubes by gently vortexing. For DNA extraction method MP.1 the DD treponeme spiked faecal samples were treated as 'wet soil samples' (Troubleshooting section of Manufacturer's protocol) where the 250 mg starting amount of DD treponeme spiked faecal samples were first centrifuged in the PowerBead Tubes, with the PowerBeads and solution removed beforehand, at 10 000 *g* for 30 seconds before discarding as much supernatant as possible. The PowerBeads and solution were then added back into the PowerBead Tubes, vortexed gently to mix and the manufacturer's protocol was then followed as described below.

To enable complete cell lysis, 60 µl of Solution C1 was added to the PowerBead Tubes and vortexed briefly. For DNA extraction method MP.2 only, the alternative lysis protocol from the Troubleshooting section of the manufacturer's protocol was then followed where the PowerBead Tubes were vortexed for 3-4 seconds followed by incubation at 70°C for 5 minutes. These two steps were repeated a second time followed by a final vortex of 3-4 seconds before proceeding with the next step of the standard manufacturer's protocol described below.

The PowerBead Tubes were then vortexed horizontally for 10 minutes on maximum vortex speed followed by centrifuging at 10 000 *g* for 30 seconds. The supernatants were transferred to 2 ml collection tubes, to which 250 µl of Solution C2 was added. The tubes were then mixed by vortexing for 5 seconds before incubating at 4 °C for 5 minutes, followed by centrifuging at 10 000 *g* for 1 minute. Up to 600 µl of each supernatant was transferred to a new 2 ml collection tubes, discarding the pellets, and 200 µl of Solution C3 was added to the supernatants. The supernatants were then incubated at 4°C for 5 minutes before centrifuging at 10 000 *g* for 1 minute. Up to 750 µl of each supernatant was then

transferred to new 2 ml collection tubes and 1.2 ml of Solution C4 (shaken to mix prior to use) was added.

The supernatants were then vortexed for 5 seconds before transferring 675 µl onto the Spin Filters provided. Spin Filters were centrifuged for at 10 000 *g* for 1 minute and the filtrate discarded. This process was repeated until all the remaining supernatant was loaded onto the Spin Filters for each sample.

The Spin Filters were then washed by adding 500 µl of Solution C5 and centrifuging at 10 000 *g* for 30 seconds and the filtrate was discarded. The Spin Filters were then further centrifuged at 10 000 *g* for 1 minute, the filtrate discarded and the Spin Filters placed into new 2 ml collection tubes. To the centre of the Spin Filter membranes, 100 µl of Solution C6 was added followed by centrifuging at 10 000 *g* for 30 seconds to enable DNA elution. The Spin Filter was then discarded and the DNA eluted stored at -20°C.

PowerFecal° DNA Isolation Kit (PF; MO BIO laboratories Inc, Carlsbad, CA, USA)

A starting amount of 250 mg of each DD treponeme spiked faecal sample was added to individual Dry Bead Tubes provided with the kit and 750 µl of Bead Solution was then added to each tube and vortexed gently to mix. To assist cell lysis, 60 µl of Solution C1 was added to each tube and vortexed briefly followed by incubation at 65°C for 10 minutes. The tubes were then vortexed horizontally at maximum speed for 10 minutes before centrifuging at 13 000 *g* for 1 minute.

The supernatants were transferred to new 2 ml collection tubes and 250µl of Solution C2 was added and mixed by vortexing briefly. Tubes were then incubated at 4°C for 5 minutes followed by centrifuging at 13 000 *g* for 1 minute. The supernatants (up to 600 µl) were transferred to new 2 ml collection tubes and 200 µl of Solution C3 was added. The tubes were then vortexed briefly and incubated at 4°C for 5 minutes before centrifuging at 13 000 *g* for 1 minute. Up to 750 µl of each supernatant was transferred to a new collection tube, to which 1.2 ml of Solution C4 (shaken before use) was added and the mixtures vortexed for 5 seconds.

The Spin Filters provided were then loaded with 650µl of supernatant and centrifuged at 13 000 *g* for 1 minute. The filtrates were discarded and the process repeated until all the remaining supernatant for each sample had been loaded onto the individual Spin Filters.

The Spin Filters were then washed by adding 500 µl of Solution C5 and centrifuging at 13 000 *g* for 1 minute. The filtrate was then discarded and the Spin Filters centrifuged a second time and placed into new 2 ml collection tubes.

To the centre of the Spin Filter membranes, 100 µl of Solution C6 was added to enable DNA elution from the membranes. The Spin Filters were then centrifuged at 13 000 *g* for 1 minute and the eluted DNA in the collection tubes were stored at -20°C.

Stool DNA Isolation Kit (BN; Norgen Biotek Corp, Thorold, Canada)

For each DD treponeme spiked faecal sample a starting amount of 100 mg, 150 mg or 200mg was added to the Bead Tubes provided along with 1 ml of Lysis Buffer L and briefly vortexed before adding Lysis Additive A and briefly vortexing again. The Bead Tubes were then vortexed horizontally on maximum speed for 3 minutes followed by centrifuging at 14 000 *g* for 2 minutes. The supernatants (up to 600 µl) were transferred to new 1.5 ml microcentrifuge tubes where 100 µl of Binding Buffer I was added, mixed by inverting and incubated on ice for 10 minutes. The cell lysates were then centrifuged in the microcentrifuge tubes at 14 000 *g* for 2 minutes to pellet the cell debris and up to 700 µl of the supernatants were transferred to new 2 ml microcentrifuge tubes . An equal volume of 70% molecular grade ethanol was then added and the tubes were vortexed briefly.

To spin columns placed in collection tubes, 600 µl of each clarified lysate and ethanol mixture were added and then centrifuged at 3 500 *g* for 1 minute. The filtrate was discarded and the process repeated with the remaining clarified lysate mixture for each sample.

The spin columns were then washed by adding 500 µl of Buffer SK and centrifuging at 14 000 *g* for 1 minute. The filtrate was discarded and 500 µl of Wash Solution A was added to the spin columns followed by centrifuging at 14 000 *g* for 1 minute. The filtrate was discarded and the Wash Solution A step was repeated. The spin columns were centrifuged at 14 000 *g* for a further 2 minutes to ensure the resin had dried.

The spin columns were placed in the 1.7 ml Elution Tubes provided and 50 µl of Elution Buffer B was added to the columns before centrifuging at 200 *g* for 2 minutes, followed by centrifuging at 14 000 *g* for 1 minute. The spin columns were then discarded and the DNA elute stored at -20°C.

Appendix B

Supplementary material relating to Chapter 5

On the next few pages are split decomposition analysis graphs relating to STs belonging to the *T. medium, T. phagedenis* and *T. pedis* DD treponeme phylogroups obtained from MLST of tissue and tissue culture samples.

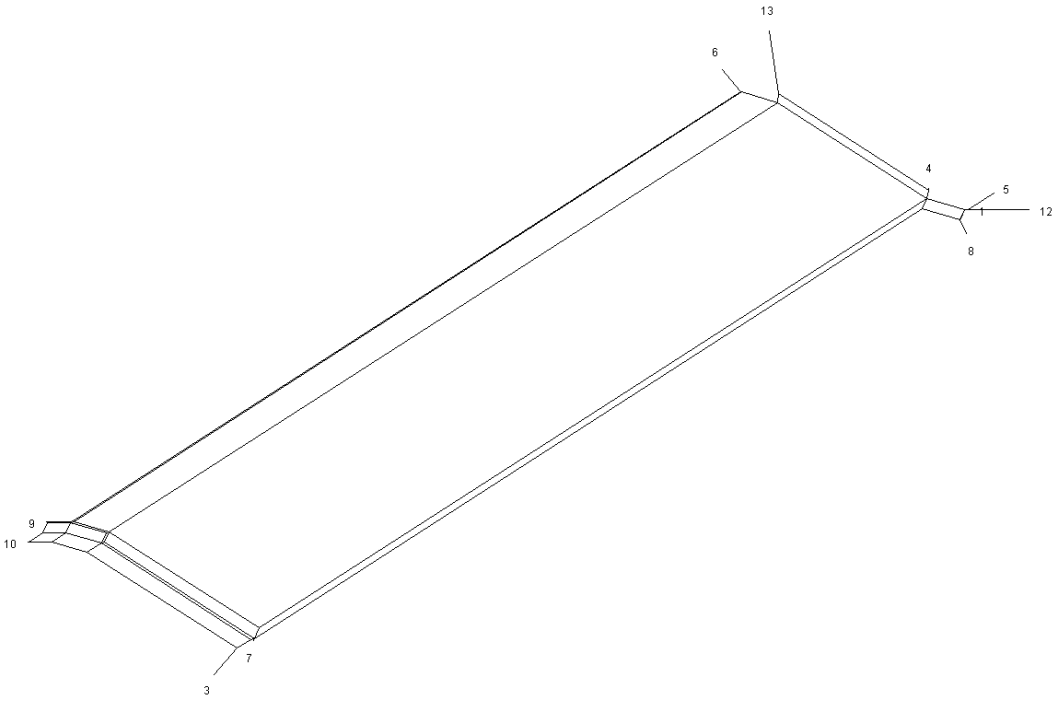


Figure B.1: Splits decomposition analysis of T. medium phylogroup STs

Numbers refer to ST, each represented by one isolate: 1) T. medium phylogroup strain T18; 2) T. medium ATCC 700293; 3) T. medium phylogroup strain 7.45G; 4) T. medium phylogroup strain T136; 5) T. medium phylogroup strain T52; 6) T. medium phylogroup strain OV11F; 7) T. medium phylogroup strain EL022; 8) T. medium phylogroup strain T380; 9) T. medium phylogroup strain 3E; 10) T. medium phylogroup strain EL024; 12) 490T and 13) 495T.

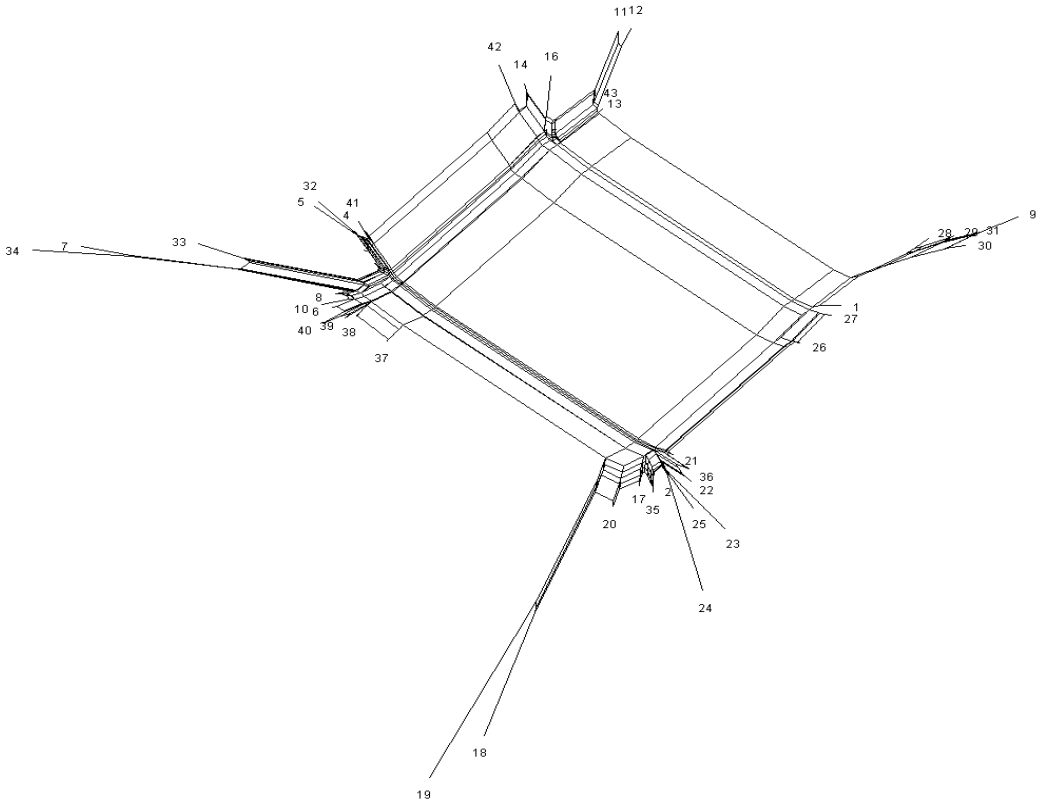


Figure B.2: Splits decomposition analysis of T. phagedenis phylogroup STs

Numbers refer to ST, each represented by one isolate: 1) T. phagedenis phylogroup strain T320A; 2) T. phagedenis phylogroup strain1498 MED AG; 3) T. phagedenis phylogroup strain T100A; 4) T. phagedenis phylogroup strain T2723; 5) T. phagedenis phylogroup strain T2721A; 6) T. phagedenis phylogroup strain DD3F; 7) T. phagedenis Reiter; 8) T. phagedenis phylogroup strain G169A; 9) T. phagedenis phylogroup strain ST27; 10) T. phagedenis phylogroup strain T119A; 11) T. phagedenis phylogroup strain G2S4F; 12) T. phagedenis phylogroup strain SL2; 13) T. phagedenis phylogroup strain G10JD; 14) T. phagedenis phylogroup strain T645C; 15) T. phagedenis phylogroup strain 2LC; 16) T. phagedenis phylogroup strain S3R; 17) T. phagedenis phylogroup strain 11A; 18) T. phagedenis ATCC Kazan 8; 19) T. phagedenis CIP; 20) T. phagedenis phylogroup strain K; 21) T. phagedenis phylogroup strain DD2R; 22) T. phagedenis phylogroup strain DD2F; 23) T. phagedenis phylogroup strain EL022; 24) T. phagedenis phylogroup strain W35D; 25) T. phagedenis phylogroup strain DD1R; 26) T. phagedenis phylogroup strain T200; 27) T. phagedenis phylogroup strain T52; 28) T. phagedenis phylogroup strain T116; 29) T. phagedenis phylogroup strain G2SL5; 30) T. phagedenis phylogroup strain ST25; 31) T. phagedenis phylogroup strain ST24; 32) T. phagedenis phylogroup strain DD1F; 33) T. phagedenis 4A; 34) T. phagedenis F4021; 35) T. phagedenis phylogroup V1; 36) 490T; 37) 1C; 38) 568C; 39) 321.1C; 40) 321C; 41) 325C; 42) 325.1C and 43) 253C.

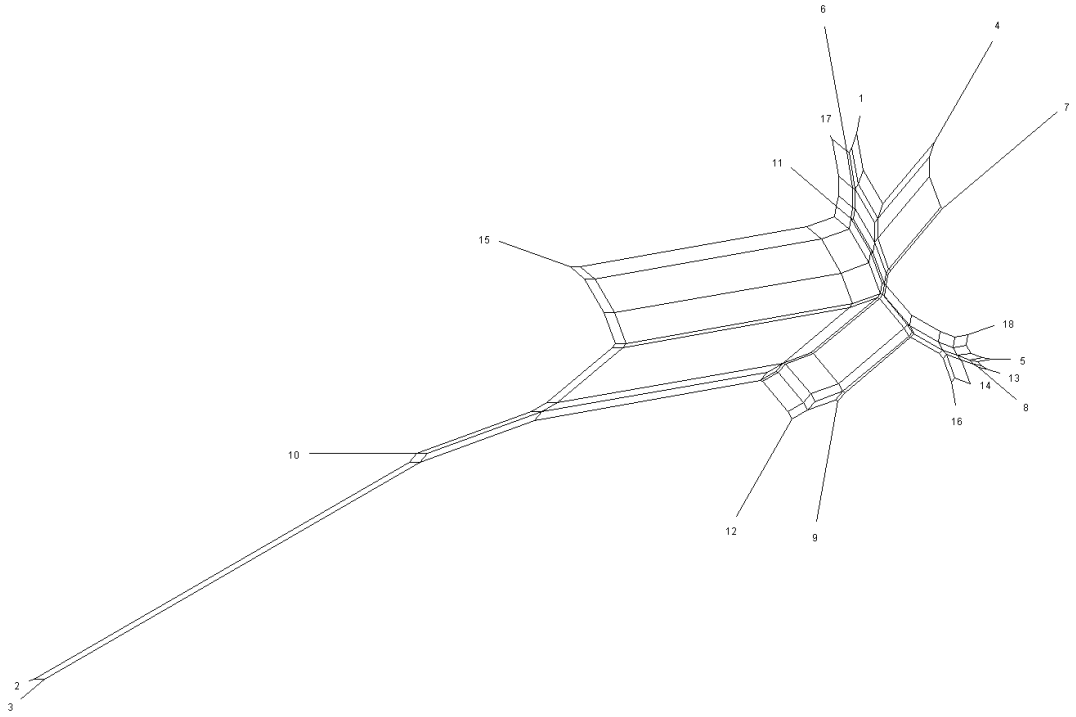


Figure B.3: Splits decomposition analysis of T. pedis phylogroup STs

Numbers refer to ST, each represented by one isolate: 1) T. pedis phylogroup strain T3552B; 2) T. pedis phylogroup strain G3T1; 3) T. pedis phylogroup strain G2JD; 4) T. pedis phylogroup strain 9185 Med Ag 2; 5) T. pedis phylogroup strain Ovine G179; 6) T. pedis phylogroup strain T3551c; 7) T. pedis phylogroup strain T A4; 8) 517C; 9) 452T; 10) 573T; 11) 721T; 12) 494T; 13) 495T; 14) 490T; 15) 688C; 16) 204C and 17) 60C.

Appendix C

Supplementary material relating to Chapter 6

On the next few pages are tables of growth and motility scores relating to temperature, faecal and bedding microcosms described in Chapter 6.

Table C.1: Median growth scores of DD treponemes subjected to different temperatures[a]

Temperature	Strain no.[b]	Culture type[c]	Number of days incubation in microcosm						
			0	2	4	7	10	15	21
4°C	T19	Microcosm	2	2	1	2	2	2	2
		Sub-culture	5	5	5	5	5	4	4
	T320A	Microcosm	2	2	2	3	3	2	2
		Sub-culture	5	5	5	5	5	5	5
	T3552B	Microcosm	2	2	2	3	3	3	2
		Sub-culture	5	5	5	5	5	4.5	1
12°C	T19	Microcosm	2	2	1	2	2	2	2
		Sub-culture	5	5	5	5	5	3	0
	T320A	Microcosm	2	2	2	3	2	2	2
		Sub-culture	5	5	5	4	5	5	5
	T3552B	Microcosm	3	3	3	3	3	2	2
		Sub-culture	5	5	4	5	4	4	4
20°C	T19	Microcosm	2	2	2	2	2	1	1
		Sub-culture	5	5	5	5	0	0	0
	T320A	Microcosm	2	2	2	2	2.5	3	3
		Sub-culture	5	4.5	5	5	5	4.5	5
	T3552B	Microcosm	3	3	2	2	2	1.5	1.5
		Sub-culture	5	5	4	4.5	5	4	5
37°C	T19	Microcosm	2	3	4	5	5	5	5
		Sub-culture	5	5	5	5	5	5	5
	T320A	Microcosm	2	4	4	5	5	4	4
		Sub-culture	5	5	5	5	5	5	5
	T3552B	Microcosm	2	4	4	5	5	4	5
		Sub-culture	5	5	5	4	5	4	5
45°C	T19	Microcosm	2	2	2	2	2	2	2
		Sub-culture	5	0	0	0	0	0	0
	T320A	Microcosm	2	2	2	2	2	1	2
		Sub-culture	5	4	0	0	0	0	0
	T3552B	Microcosm	2	2	2	3	2	2	2
		Sub-culture	5	1	0	0	0	0	0
60°C	T19	Microcosm	2	2	2	1	NT	NT	NT
		Sub-culture	5	0	0	0	NT	NT	NT
	T320A	Microcosm	3	2	2	1.5	NT	NT	NT
		Sub-culture	5	0	0	0	NT	NT	NT
	T3552B	Microcosm	3	3	2	2	NT	NT	NT
		Sub-culture	5	0	0	0	NT	NT	NT

[a] *Growth scores of 0-5 with 0 = no growth and 5 = dense growth. NT denotes not tested*.

[b] *T19 belongs to the T. medium DD treponeme phylogroup, T320A belong to the T. phagedenis DD treponeme phylogroup and T3552B belong to the T. pedis DD treponeme phylogroup.*

[c] *For sub-culture the growth scores from 7 days incubation are displayed.*

Table C.2: Median motility scores of DD treponemes subjected to different temperatures[a]

			Number of days incubation in microcosm						
Temperature	**Strain no.[b]**	**Culture type[c]**	**0**	**2**	**4**	**7**	**10**	**15**	**21**
4°C	**T19**	**Microcosm**	4	3	4	2	2	1	1
		Sub-culture	4	4	3	3	4	4	4
	T320A	**Microcosm**	3	3	1	1	2	1	2
		Sub-culture	5	4	4	4	4	4	4
	T3552B	**Microcosm**	3	2	1	1	1	1	1
		Sub-culture	2	3	2	1	2	1.5	1
12°C	**T19**	**Microcosm**	4	3	3	2	2	2	1
		Sub-culture	4	4	4	4	3	3	0
	T320A	**Microcosm**	3	2	2	1	2	1	1
		Sub-culture	4	5	4	3	3	4	3
	T3552B	**Microcosm**	3	1	1	1	2	1	1
		Sub-culture	1	1	1	2	2	3	2
20°C	**T19**	**Microcosm**	4	2	2	2	1	0	0
		Sub-culture	4	4	3	3	0	0	0
	T320A	**Microcosm**	3.5	2.5	1.5	4.5	4.5	4.5	3
		Sub-culture	5	4	4	3	4	4	3
	T3552B	**Microcosm**	2	1	1	1	1	1	1
		Sub-culture	2	2	1	2	1.5	2	1
37°C	**T19**	**Microcosm**	4	4	3	3	2	1	1
		Sub-culture	4	2	2	2	2	3	2
	T320A	**Microcosm**	3	3	4	4	2	1	3
		Sub-culture	4	4	3	1	3	4	3
	T3552B	**Microcosm**	2	2	2	2	2	1	1
		Sub-culture	2	2	2	1	1	2	1
45°C	**T19**	**Microcosm**	3	0	0	0	1	1	1
		Sub-culture	4	0	0	0	0	0	0
	T320A	**Microcosm**	3	1	0	1	0	1	1
		Sub-culture	5	5	0	0	0	0	0
	T3552B	**Microcosm**	2	1	1	1	1	1	1
		Sub-culture	2	1	0	0	0	0	0
60°C	**T19**	**Microcosm**	2	0	1	1	NT	NT	NT
		Sub-culture	4	0	0	0	NT	NT	NT
	T320A	**Microcosm**	4	1	1	0	NT	NT	NT
		Sub-culture	3	0	0	0	NT	NT	NT
	T3552B	**Microcosm**	2	1	1	1	NT	NT	NT
		Sub-culture	1	0	0	0	NT	NT	NT

[a] Motility scores of 0-5 with 0 = no motility and 5 = 100% motility. NT denotes not tested.

[b] T19 belongs to the T. medium DD treponeme phylogroup, T320A belong to the T. phagedenis DD treponeme phylogroup and T3552B belong to the T. pedis DD treponeme phylogroup.

[c] For sub-culture the motility scores from 7 days incubation are displayed.

Table C.3: Median growth scores of DD treponemes incubated in bovine faecal microcosms[a]

Strain no.[b]	Culture type[c]	Number of days incubation in microcosm							
		0	1	2	3	4	5	6	7
T19	Microcosm	3	3	3	3	3	3	3	3
	Sub-culture	3	1	0	1	1	1	1	0
T19 Positve control	Microcosm	3	3	3	3	3	3	3	3
	Sub-culture	2	1	1	1	1	1	1	1
T320A	Microcosm	4	4	4	4	4	4	4	4
	Sub-culture	5	4	1	1	1	1	1	1
T320A Positve control	Microcosm	4	4	4	4	4	4	4	4
	Sub-culture	5	5	5	5	4	4	2	1
T3552B	Microcosm	4	4	4	4	4	4	4	4
	Sub-culture	5	2	1	1	1	2	1	1
T3552B Positve control	Microcosm	4	4	4	4	4	4	4	4
	Sub-culture	5	4	2	2	2	2	2	1

[a] Growth scores of 0-5 with 0 = no growth and 5 = dense growth.

[b] T19 belongs to the T. medium DD treponeme phylogroup, T320A belong to the T. phagedenis DD treponeme phylogroup and T3552B belong to the T. pedis DD treponeme phylogroup.

[c] For sub-culture the growth scores from 7 days incubation are displayed.

Table C.4: Median motility scores of DD treponemes incubated in bovine faecal microcosms[a]

Strain no.[b]	Culture type[c]	Number of days incubation in microcosm							
		0	1	2	3	4	5	6	7
T19	Microcosm	1	1	1	1	1	1	1	1
	Sub-culture	3	1	0	0.5	0	0	0	0
T19 positive control	Microcosm	1	1	1	1	1	1	1	1
	Sub-culture	3	1	0	1	0	0	0	0
T320A	Microcosm	1	1	1	1	1	1	1	1
	Sub-culture	4	4	1	1	1	1	1	1
T320A Positive control	Microcosm	3	3	2	1	1	1	1	1
	Sub-culture	3	4	3	4	4	3	3	1
T3552B	Microcosm	1	1	1	1	1	1	1	1
	Sub-culture	3	1	1	1	1	1	1	0
T3552B Positive control	Microcosm	2	2	1	1	1	1	1	1
	Sub-culture	3	3	1	1	1	1	1	0

[a] Motility scores of 0-5 with 0 = no motility and 5 = 100% motility.

[b] T19 belongs to the T. medium DD treponeme phylogroup, T320A belong to the T. phagedenis DD treponeme phylogroup and T3552B belong to the T. pedis DD treponeme phylogroup.

[c] For sub-culture the motility scores from 7 days incubation are displayed.

Table C.5: Median growth scores of DD treponemes incubated in bedding microcosms[a]

Type of Bedding	Culture type[b]	Number of days incubation in microcosm							
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Straw	**Microcosm**	2	1	1	1	1	1	1	1
	Sub-culture	1	1	1	1	0	1	0	0
Sawdust	**Microcosm**	4	3	3	3	3	3	3	2
	Sub-culture	2	2	2	2	1.5	2	2	1
RMS	**Microcosm**	4	3	3	3	3	3	3	2
	Sub-culture	3	2	2	2	2	2	1	1
Sand	**Microcosm**	4	4	4	4	4	4	4	4
	Sub-culture	3	3	3	3	3	3	3	3
Sand + 5% lime	**Microcosm**	0	0	0	0	0	0	0	0
	Sub-culture	0.5	0	0	0	0	0	0	0
Control	**Microcosm**	4	4	4	4	4	4	4	4
	Sub-culture	5	4.5	5	3.5	3	2	1.5	2

[a] Growth scores of 0-5 with 0 = no growth and 5 = dense growth. T. phagedenis DD treponeme phylogroup strain T320A only investigated.

[b] For sub-culture the growth scores from 7 days incubation are displayed.

Table C.6: Median motility scores of DD treponemes incubated in bedding microcosms[a]

Type of Bedding	Culture type[b]	Number of days incubation in microcosm							
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Straw	**Microcosm**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
	Sub-culture	**1**	**1**	**1**	**0.5**	**0**	**1**	**0**	**0**
Sawdust	**Microcosm**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
	Sub-culture	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
RMS	**Microcosm**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
	Sub-culture	**4**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
Sand	**Microcosm**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
	Sub-culture	**2**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
Sand + 5% lime	**Microcosm**	0	**0**	**0**	**0**	**0**	**0**	**0**	0
	Sub-culture	0	**0**	**0**	**0**	**0**	**0**	**0**	0
Control	**Microcosm**	**2**	**2**	**1**	**1**	**1**	**1**	**1**	**1**
	Sub-culture	**4.3**	**3.5**	**4**	**2.5**	**2.5**	**1**	**0.5**	**0.5**

[a] Motility scores of 0-5 with 0 = no motility and 5 = 100% motility. T. phagedenis DD treponeme phylogroup strain T320A only investigated.

[b] For sub-culture the growth scores from 7 days incubation are displayed.